

PRODUCTION OF EXTRACELLULAR POLYSACCHA-
RIDE BY ZOOGLOEA RAMIGERA AND ITS USE AS
AN ADSORBING AGENT FOR HEAVY METALS

BY

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Department of Applied Microbiology

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Title and subtitle Production of extracellular polysaccharide by <u>Zoogloea ramigera</u> and its use as an adsorbing agent for heavy metals.			
Abstract Extracellular polysaccharides produced by bacteria are generally secreted into the growth medium. The presence of polysaccharides in the cultivation medium have a striking effect on mass transfer. As synthesis of polysaccharides by bacteria, with some exceptions, is dependent on progressive energy metabolism, enough oxygen must be transferred to the cells during the production phase. Thus, reasonable levels of dissolved oxygen in viscous cultivation broths, demands high energy input. Production of extracellular polysaccharide by <u>Zoogloea ramigera</u> manifests a more favourable pattern regarding the conditions governing oxygen transfer. The polysaccharides formed during the synthesis phase are anchored to the surfaces of the cells as capsules. When the polysaccharides are attached to the cells the viscosity of the broth is low which allows high oxygen transfer rates to be maintained. When the capsular material is detached from the cells the viscosity of the broth increases sharply. But as the demand for oxygen is negligible during the release phase it is not necessary to maintain the oxygen transfer rate at a high level during this phase. <u>Z. ramigera</u> exhibits sensitivity towards heavy metals. Low concentrations of cadmium, cobalt, uranium and zinc strongly affect the growth of the organism. Biomass, consisting of bacterial cells and polysaccharide produced by the organism is used as an adsorbing agent for heavy metals in a system which allows regeneration and reuse of biomass. Selective accumulation of cadmium, copper and uranium can be performed through regulation of pH. The uptake of cadmium and copper by <u>Z. ramigera</u> was compared to metal uptake by polyacrylic acid, carboxymethylcellulose and <u>Pseudomonas denitrificans</u> . <u>Z. ramigera</u> was superior to the other agents tested. A continuous system for retrieval of copper by biomass produced by <u>Z. ramigera</u> is discussed. Metal uptake has been measured by atomic absorption spectrophotometric methods or by potentiometric methods.			
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LIST OF PAPERS

This thesis consists of the following papers referred to by Roman numerals in the text.

- I Norberg, A B and Enfors, S-O. Production of extracellular polysaccharide by Zoogloea ramigera. Appl. Environ. Microbiol., 44, 1231 (1982).
- II Norberg, A B and Molin, N. Toxicity of cadmium, cobalt, uranium and zinc to Zoogloea ramigera. Water Res., in press.
- III Norberg, A B and Persson, H. Accumulation of heavy metal ions by Zoogloea ramigera. Accepted for publication in Biotech. Bioeng.
- IV Norberg, A B and Molin, N. Selective accumulation of heavy metal ions by Zoogloea ramigera. (Submitted)
- V Norberg, A B and Persson, H. Heavy metal adsorption by Zoogloea ramigera in comparison to other adsorbing agents. (Submitted)
- VI Norberg, A B and Rydin, S. Development of a continuous process for metal accumulation by Zoogloea ramigera. (Submitted)
- VII Norberg, A B, Molin, N and Wiberger, I. Svensk patentansökan Tillverkning och användning av biopolymer.

A MICROBIAL EXOPOLYSACCHARIDES - PRODUCTION AND PROPERTIES

INTRODUCTION

Carbon source supplied to microorganisms can be converted into a wide spectrum of different products in anabolic and catabolic pathways. Polysaccharide is a group of products assembled through anabolic processes which utilize ATP energy obtained through catabolic pathways. However the anabolic fate of the substrate can still take anyone of several directions. They can be integrated in components of cell structures, such as the peptidoglycan or murein present in both Gram-negative and Gram-positive cells. In addition to the peptidoglycan bacterial cell walls contain a number of accessory polymers. Teichoic acids are found in Gram-positive bacteria and lipopolysaccharides are an integral part of Gram-negative cells.

The substrate can also be directed to intracellular polymer synthesis. Glycogen is commonly found in bacteria where it can act as an energy and carbon source for future use.

In addition numerous bacteria synthesize polysaccharides which are found outside the cell attached to the cell wall or secreted into the surrounding medium.

Depending on their structural relationship to the bacterial cell wall they have been variously termed slime or capsular polysaccharides. The name exopolysaccharide used in this survey provides a general term for all these forms of bacterial polysaccharides found outside the bacterial cell wall.

Furthermore exopolysaccharides can occur in different forms: either attached to the microbial cell wall in the form of a capsule or found throughout the cultivation medium in the form of dispersed or soluble slime. The two forms can be distinguished by negative staining techniques (1).

Exopolysaccharides from pathogenic organisms, i.e., Brucella, Neisseria and Escherichia, are generally not important for technical purposes and are therefore not considered in the present review.

COMPOSITION OF MICROBIAL EXOPOLYSACCHARIDES

Bacterial exopolysaccharides can be divided into homopolysaccharides, which are composed of only one type of monomeric unit, and heteropolysaccharides which contain two or more different monomeric units.

Neutral monosaccharides appear to be the most common building block of bacterial exopolysaccharides. Among the sugars hexoses such as fructose, galactose, glucose and mannose occur as components in bacterial exopolysaccharides. Less abundant in exopolysaccharides are the pentoses arabinose, ribose and xylose.

Derivatives of monosaccharides are common in exopolysaccharides. The most important are sugar acids. They can occur in three different shapes aldonic, aldarcic or uronic acids depending on where the aldose is oxidised. Uronic acids, such as glucuronic acid, galacturonic acid and mannuronic acid, are commonly found in bacterial exopolysaccharides where they contribute to the negative charge of the polymer. Glucuronic acid is most abundantly found in exopolysaccharides from bacteria.

The anionic character of bacterial exopolysaccharides can be derived from other components incorporated in the polymer chain. Pyruvate linked as a ketal to neutral monosaccharides is frequently found in exopolysaccharides produced by bacteria.

The carboxylic group of pyruvate is free and can thus contribute to the negative charge of the polysaccharide.

Another non-sugar component of exopolysaccharides is acetate. Furthermore acetate, formate and succinate can occasionally be included in polysaccharides. These acyl groups are mainly linked to one of the many available hydroxyl groups on the incorporated sugars.

Some heteropolysaccharides contain inorganic substituents. The most important substituent is phosphate which is a component of phosphomannan produced by fungi.

EXOPOLYSACCHARIDE SYNTHESIS

Synthesis outside the cell

Extracellular polysaccharide synthesis mediated by enzymes distinctly separated from the cells but of microbial origin takes place in a few but well-known examples. Dextran and levan are synthesized in this manner. The name dextran was coined as early as 1869 by Scheibler (2). Dextran is a homopolysaccharide composed of glucose.

In 1941 cell-free enzymic synthesis of dextran was demonstrated by Hehre (3). The enzyme responsible for the polymerization was named dextransucrase by Hestrin (4). Sucrose acts as a substrate for the enzyme and furthermore initiates its own polymerization by acting as an acceptor molecule. The glucose units are linked predominantly α 1,6 but α 1,3 and α 1,4 linkages also exist. The degree of branching varies depending on the bacterial strain used. When branched dextran is produced it is considered to be due to the presence of more than one enzyme (5).

Levan production, catalyzed by levansucrase, is considered to be synthesized in a way analog to dextran. However the repeating unit in levan is fructose. The linkages in levan are β 2,6 and β 2,1.

Both levan and dextran are formed from sucrose and they do not require input of energy in the form of activated phosphorylated sugars in their synthesis (6).

Synthesis requiring active cells

The majority of the microbial exopolysaccharides are synthesized in a manner which differs considerably from the one described above. The synthesis of most polysaccharides requires the presence of a considerable number of enzymes. These enzymes are involved in the uptake of substrate by the microbial cell and in the modification of the substrate which takes place inside the cell and in the polymerization of the components of the polysaccharide and finally in the excretion of the polymer into the surrounding medium.

The formation of building blocks for a homo- or a heteropolysaccharide, using glucose as substrate, involves a series of enzymatic processes. Some of these initial reactions are common to both the anabolic and catabolic fate of the substrate. The steps in mind are conversion of glucose to glucose-1-P via glucose-6-P. If the substrate is further catabolized, and thus used for energy production, glucose-6-P will proceed through the glycolysis, enter the TCA cycle and reach the respiratory chain where NADH is oxidized and energy rich ATP is formed. However, if the exopolysaccharide contains pyruvate or acetate formation of these components will take place during the catabolism of glucose. These metabolites can be incorporated into the polymer chain at a later stage.

The biosynthetic pathways leading from a simple substrate such as glucose-6-P to its coupling to an end of the polysaccharid chain involves a consecutive number of reactions starting with the formation of glucose-1-P which reacts with a nucleoside triphosphate forming a nucleoside diphosphate glucose. The nucleoside diphosphate, such as UDP, serves both as a sugar carrier and as a donor of sugars in the synthesis of polysaccharides. UDP, although most common, is not the only nucleoside diphosphate in bacterial cells. Guanosine diphosphate, GDP, is another important sugar carrier which can carry mannose derived from the conversion of glucose.

The sugar nucleotides provide a mechanism for sugar interconversions. NDP-sugars such as GDP-mannose can be reduced to GDP-fucose and GDP-rhamnose which are components of bacterial lipopolysaccharides found in cell walls as well as building blocks for exopolysaccharides. Another common part in bacterial exopolysaccharides is glucuronic acid. This molecule is formed through oxidation of UDP-glucose. Furthermore UDP-glucose can be reversibly converted into UDP-galactose.

Apart from these sugar interconversions the nucleotides also serve another biochemical function as activators of monosaccharides to admit transfer of the sugars to special acceptor molecules.

The next step in the formation of exopolysaccharides is the transfer of monosaccharides or derivatives of monosaccharides from the sugar nucleotides to an isoprenoid lipid (7). This lipid also acts as an acceptor for building blocks involved in the synthesis of the bacterial wall. The lipid is located in the cell membrane whereas the formation of sugar nucleotides takes place within the cell.

Many exopolysaccharides contain organic substituents, such as acetate and pyruvate, but little is known about their incorporation. Acetyl-CoA can be the donor for acetate and it is probable that phosphoenolpyruvate is the donor for pyruvate.

Whether these compounds are incorporated while the polymer chain is attached to the lipid or after the release of the polysaccharide from the lipid is not clear. The reactions involved in the release of the polysaccharide from the isoprenoid lipid are not fully understood. If the polysaccharide chain should be integrated in a capsule it could be transferred to an acceptor molecule at the cell wall. Slime producing organisms may excrete the polymer chain into the surrounding medium.

MICROORGANISMS PRODUCING EXOPOLYSACCHARIDES

Homopolysaccharides

Exopolysaccharides are produced by a wide spectrum of microorganisms. Only a few of these species will be considered in this survey. An important factor determining the occurrence in natural habitats of microbial exopolysaccharides is the availability of suitable substrates under environmental conditions that support microbial growth. Most of the dextrans are produced from sucrose by the nonpathogenic bacteria Leuconostoc mesenteroides and L. dextranicum. Other bacteria producing dextran from sucrose are certain species within the genera Streptococcus and Lactobacillus. The glucose units are mainly linked by 1,6 bonds, but 1,3 and 1,2 linkages also occur. The degree of branching depends primarily on the strain that produces the polymer. L. mesenteroides, and particularly the strain NRRL B-512 F, synthesize a dextran which is predominantly linked with 1,6 bonds (95 %) the remainder of which are branches linked at C-3 positions (8). Dextrans obtained from other sources usually show a higher degree of branching.

Levan, a polyfructose, is synthesized from sucrose by the enzyme levansucrase. The polymerization can occur in cell-free solutions. Many sources exist for the production of levan. Aerobacter aerogenes, Azotobacter chroococcum, Bacillus spp, Pseudomonas fluorescens and Streptococcus salivarius are some of the bacteria which have been described as levan producers.

Another homopolysaccharide, synthesized by cell-associated enzymes, is cellulose. It is produced by Acetobacter xylinum.

Curdlan, a 1,3 linked glucan, is produced by Alcaligenes faecalis var. myxogenes (9). The carbon substrate in this process is ethylene glycol.

Heteropolysaccharides

Among the heteropolysaccharide producers Xanthomonas campestris is the most well-known. The polymer produced is xanthan. The bacterium is a plant pathogen blocking water movement within the plant. X. campestris is known to produce vascular disease in cabbages. Xanthan is composed of glucose, mannose, glucuronate, acetate and pyruvate.

Alginate originally obtained from brown seaweed, such as Laminaria, Nereocystis and Macrocystis, is composed of mannuronate and guluronate. During recent decades, certain bacteria have been found to produce exopolysaccharides with the same structural units (10). The organisms used are Pseudomonas aeruginosa and Azotobacter vinelandii.

Hyaluronic acid, which is a heteropolysaccharide composed of N-acetylglucosamine - a constituent of bacterial cell walls, and glucuronate can be produced from Streptococcus spp.

To solidify microbiological media agar is added as it has excellent gel-forming properties. Agar is a heteropolysaccharide traditionally extracted from red algae such as Gracilaria. The polysaccharide contains galactose, sulphate and to some extent pyruvate. Kappa-carrageenan is another heteropolysaccharide extracted from red algae. Kappa-carrageenan differs from agar in that it requires a monovalent cation for gel formation. Recently a heteropolysaccharide of bacterial origin has been isolated (11). The polysaccharide has properties resembling agar and kappa-carrageenan. It is composed of glucose, rhamnose, glucuronate and acetate. The deacetylated polysaccharide produced a firm and optically clear gel. A Pseudomonas species, isolated from plant tissue, was used as producer and it is pointed out that the polymer is an excellent alternative gelling agent to agar.

Zoogloea ramigera is an interesting source for polysaccharide production. The polymer was recently revealed to be composed of glucose, galactose and pyruvate (12). Production of exopolysaccharide by Z. ramigera has been studied by Parsons and Dugan (13). The matrix has been suggested as an agent for metal adsorption (14).

Fungal polysaccharides occur in nature. For example species of Sclerotium synthesize a homopolysaccharide composed of glucose (15). Aureobasidium pullulans also produces a homopolysaccharide composed of glucose (16). A heteropolysaccharide, composed of mannose and phosphorous, is produced by Hansenula holstii (17).

PROCESS PARAMETERS INVOLVED IN THE CULTIVATION OF MICRO-ORGANISMS PRODUCING EXOPOLYSACCHARIDES

Culture Media: Much emphasise has been laid upon selecting the most favourable components of the growth medium, the amount of each ingredient and furthermore postulating the optimal growth conditions by control of environmental parameters during cultivation.

Production of most bacterial exopolysaccharides, with modification to ascertain individual needs, follows a general pattern.

A traditional medium for exopolysaccharide production by microorganisms is usually made up of components from the sources listed below.

Carbohydrates: Act as the principal carbon and energy source. Glucose, sucrose or starch could be used. Enzymic hydrolyzates of corn is another source of importance when the process is scaled up from laboratory scale to production scale.

Complex nitrogen source and organic growth factors: Different cheap sources exist, such as corn steep liquor, distillers' dried solubles and casein hydrolyzate. These ingredients provide the growing culture with a nitrogen source and some carbon source. Inorganic and organic growth factors are also supplied.

Inorganic components: Potassiumphosphate has to be provided as potassium and phosphate are required for growth. Medium buffering is also accomplished by phosphate. Magnesium plays an important role in regulating the activity of enzymes involved in exopolysaccharide synthesis and must therefore be supplied in the growth medium. Trace amounts of different metal ions are usually supplied but these can be omitted if tap water is used.

Although exopolysaccharide producing organisms are not hard to cultivate in complex media, strain variations have been reported both in continuous (18-20) and in batch fermentations (21).

A step towards defined media was reported by Cadmus et al (22). A synthetic medium was used for the growth of X. campestris. The synthetic media was supplied via the inoculum media.

Several groups working with bacterial exopolysaccharides have, during recent years, reported on successful cultivations in chemically defined media. Several strains of Xanthomonas exhibited good polysaccharide production when cultivated in defined media (23). Nutritional studies on xanthan production by X. campestris (24) revealed that the organism could grow in a salts medium with sucrose or glucose as the sole carbon source.

P. aeruginosa (19) produced exopolysaccharide in continuous culture on a chemically defined medium. Polymer was here synthesized at the same rate and at a similar conversion efficiency from the carbon source as in a medium containing yeast extract. Production of extracellular polysaccharide by Z. ramigera was successfully conducted in a chemically defined medium (13).

The concept of continuous culture is a most valuable tool in media optimization. However, when continuous culturing is applied to exopolysaccharide producers problems can occur if the organism is cultivated in complex media. The stability of the organism can be reduced resulting in decreased glucose consumption. Nonmucoid variants usually appear in the fermentation broth after 5-10 culture generation times.

Using chemically defined media facilitates studies on microbial exopolysaccharide production. Evans et al (23) report on experiments with X. juglandis showing that nitrogen, phosphate and sulphate as limiting nutrients produced cultures which were all highly viscous. It was possible to produce exopolysaccharide continuously for at least 2000 h with good yields, using nitrogen and sulphate as limiting nutrients. No strain variation occurred in these experiments. Another benefit achieved when using defined media is that it admits easier recovery of a clean polysaccharide not contaminated with unknown organic compounds from complex substrates.

Pseudomonas NCIB 11264 (25) grown in a defined medium revealed increased exopolysaccharide production under conditions of nitrogen limitation and presence of excess carbon source. This behaviour was first observed in batch culture and later confirmed in continuous culture (26).

Souw and Demain (24) conclude that X. campestris synthesize xanthan best under nitrogen limitation. Excess amounts of nitrogen compounds suppressed xanthan formation while stimulating bacterial growth.

Studies on X. campestris (27) indicated that both the amount and the type of nitrogen source strongly affected the final yield of polysaccharide from glucose. Rapid production and a high yield of crude product, containing a high percentage of X. campestris cells co-precipitated with the polysaccharide, can be achieved using high initial concentrations of nitrogen sources in the fermentation medium. Reduced nitrogen concentrations result in prolonged culturing times but the final broth will contain a much smaller percentage of bacterial cells.

Oxygen availability: Very few data are available concerning the effects of aeration on exopolysaccharide production of bacteria. Rogovin et al, (28), report that xanthan production in a 20-l fermentor was reduced when excessive agitation was used in the early stages of the fermentation. Best yields and most rapid development of culture viscosities were obtained when the culture was stirred at 150 rpm for the first 48 h and then at 280 rpm for the later part of the incubation period. The optimal aeration rate was found to be 0.5 VVM, lower rates caused prolonged fermentations. The oxygen absorption rates in these experiments ranged from about 24 m moles $O_2/l \cdot h$ at the lower agitator speeds to 96 m moles $O_2/l \cdot h$ at the higher rates.

Aeration effects on production and quality of xanthan have been studied by Cadmus et al (22) in a 10-l fermentor. Maximum pyruvate content in xanthan was obtained at an air flow rate of 0.75 VVM. However, the optimum fermentor conditions for production of xanthan gum in synthetic media were an aeration rate of 1.5 VVM and an initial agitation speed of 225 rpm increased to 300 rpm after 24 h.

Moraine and Rogovin (29) conclude that air has to be supplied to the growing culture at a rate sufficient to maintain dissolved oxygen tension above 20 % air saturation. Furthermore, agitation influenced the fermentation rate at high viscosity. The specific polymer formation rate was lower at 500 rpm than at 800 rpm even if the dissolved oxygen tension was maintained at 90 % of air saturation. The authors suggested that agitation could improve mass transfer between the medium and the cells. Oxygen availability did not limit the production of exopolysaccharide by X. juglandis in continuous culture at high viscosity (23).

Other environmental factors: Medium viscosity, pH and temperature can affect both cell growth and product formation rate in exopolysaccharide fermentations. If viscosity of the fermentation medium is increased the volumetric mass transfer coefficient ($K_L a$) can be reduced drastically (30). The reduced oxygen transfer efficiency could be due to the incomplete mechanical dispersal of air bubbles.

Both temperature and reaction pH influence the exopolysaccharide formation. The conditions for optimum production of dextransucrase are pH 6.7 and temperature 24°C. On the other hand, the optimal stability and activity of the enzyme is reached at pH 5 and a temperature of 28-30°C (31).

ECOLOGICAL SIGNIFICANCE OF EXOPOLYSACCHARIDE SYNTHESIS

It seems reasonable to assume that exopolysaccharides are not essential for bacterial growth in the laboratory. Non-mucoid mutants, unable to synthesize exopolysaccharides, occur spontaneously or after the addition of mutagenic agents to the medium. An understanding of the cellular or ecological role of the exopolysaccharides could provide a clue to how production might be increased. Explanations for exopolysaccharide synthesis have included: the anchoring of the cells to the substrate (32); polymer bridging also occurs

between cells; capsules and slime provide a suitable site of efficient operation of extracellular enzymes (33); microorganisms anchored to surfaces are more metabolically active than cells freely suspended in the surrounding medium thus nutrient uptake by cells is increased by the presence of exopolysaccharides (34); exopolysaccharides can interact with cations (35). Other suggested functions of bacterial exopolysaccharides include protection against desiccation and phage attack (36).

TECHNICAL USE OF MICROBIAL EXOPOLYSACCHARIDES

Dextran was the first microbial exopolysaccharide to be produced on an industrial scale. The polysaccharide has found applications in the pharmaceutical field as a blood plasma extender. Within chemical and biochemical research crosslinked dextran and derivatives of dextran are used for purification and isolation of biochemical compounds. An agricultural use of dextran is as a protective coating for seeds. Dextran has been used in the food industry as a stabilizer of syrups, ice cream and sweets.

Xanthan gum is currently the bacterial polysaccharide considered to have the brightest industrial potential. It has found applications in the food and pharmaceutical industry. Xanthan can be used as a stabilizer of paints. However the greatest potential market for bacterial exopolysaccharides is their possible use in enhanced oil recovery. An agent for secondary oil recovery should be able to produce high viscous solutions at low concentrations. The thickening agent should have pseudoplastic flow behaviour and it must be chemically and thermally stable over time. The quality should be consistent and the agent must be cost effective. Xanthan gum easily fulfills four of these demands but it is too expensive. However, with continued increases in the price of oil, enhanced oil recovery systems, using xanthan as a thickening agent will certainly become more attractive.

B RECOVERY OF METAL IONS

INTRODUCTION

The intention of this section is to summarize the findings regarding different aspects of metal accumulation by microbial cells. Although microorganisms are capable of concentrating a variety of different metals via energy-dependent processes, energy-independent adsorption seems to have considerable advantages for process development. Metal adsorption to microorganisms will therefore be examined with special interest.

METALS ESSENTIAL FOR MICROBIAL GROWTH

Bacteria require certain metals in solution for their growth and for their ability to withstand environmental stress. Potassium specifically activates certain enzymes; magnesium is associated with ribosomes and is needed for ribosomal structure and function. Numerous other elements, including iron, zinc, manganese, cobalt, copper and molybdenum, are required by various microorganisms.

INTRACELLULAR UPTAKE OF METALS BY ACTIVE TRANSPORT

All mediated transport processes across biological membranes have three properties in common. They show, as enzymes, saturation kinetics, they exhibit specificity which enables selective concentration of ions or molecules and they can be more or less inhibited by substances structurally related to the substrate.

Uptake of essential metal ions and substrate molecules by living microorganisms occurs via active transport. These active transport systems transfer the ion or the substrate across the membrane in only one direction and against a concentration gradient. Furthermore, active transport always depends upon metabolic energy. Transport of an uncharged molecule against a concentration gradient will not occur spontaneously. Transport of ions across membranes will not take place automatically since an electrochemical

gradient then exists. Both reactions lead to systems which gain free energy. They can only proceed if they are coupled to another reaction occurring with a decline in free energy such as the hydrolysis of ATP.

DIVALENT CATION TRANSPORT SYSTEMS

Uptake of divalent cations occurs via specific transport systems which facilitate selective concentration of essential or trace metals. Magnesium for example is essential to all living cells - no other ion can be substituted for magnesium. It is the most abundant intracellular divalent cation. Silver (37) first demonstrated active transport of magnesium by E. coli. Two kinetically different magnesium transport systems exist in E. coli. One of the systems is specific for Mg^{2+} , the other exhibits a high affinity for Mg^{2+} but also has affinities for other divalent cations (38). The growth yield from assimilated magnesium is generally between 300 to 900 g dry biomass/g magnesium, even when the external Mg^{2+} concentration varies by a factor of 100,000. Magnesium is accumulated by a temperature-dependent transport system which is inhibited by certain uncoupling agents indicating that the system is energy-dependent.

Active transport systems for manganese have been found in several bacteria. The system in E. coli (39) is highly specific temperature- and energy-dependent. It is not affected by a 100,000-fold molar excess of Mg^{2+} and Ca^{2+} .

Candida utilis (40) accumulated zinc from the growth media by an energy- and temperature-dependent process. The yeast accumulated Zn^{2+} in a cyclic manner when grown in batch culture. Uptake occurred only during the lag and late-exponential phases. No uptake of zinc was observed during the early-exponential or stationary phases. Zinc-deficient cells could accumulate as much as 7.8 mg Zn^{2+} /g dry weight.

Living cells, including microorganisms, maintain low intracellular calcium ion concentration and high cytoplasmic levels of magnesium in contrast to the reverse concentration ratio in the media surrounding the cells. The hypothesis that all microbial cells actively secrete calcium by a specific energy-dependent active process was suggested by Silver and Kralovic (41). Membranes from E. coli, prepared by decompression in a French Press chamber,

yield everted membranes (42). These membranes accumulated Ca^{2+} in an energy-dependent process and they are furthermore unable to accumulate amino acids, thus demonstrating active efflux of calcium.

Saccharomyces cerevisiae (43) have been reported to accumulate cobalt and cadmium by an energy-dependent process. The intracellular concentration of Mg^{2+} was not affected by the accumulation of Co^{2+} and Cd^{2+} . Addition of Co^{2+} and Cd^{2+} instead resulted in a rapid efflux of K^{+} ions. Inhibition of Co^{2+} uptake was most pronounced by divalent cations of a similar size to Co^{2+} . The authors suggested that Co^{2+} , Ni^{2+} , Zn^{2+} and Mn^{2+} shared a cation uptake system of low specificity. Uptake of uranyl ions by actively growing filamentous fungi has been demonstrated (46). Fungal growth was not inhibited by uranium over the range 100 to 1,000 ppm. The organism could accumulate 0.17 g uranium/g dry weight of mycelium.

MONOVALENT CATION TRANSPORT SYSTEMS

The potassium requirement for the growth of microorganisms corresponds to a growth yield of approximately 60 g dry biomass/g potassium. Several kinetically and genetically different potassium transport systems exist in E. coli (44). Other alkali metal ions can be competitive substrates for the potassium transport system. Thallium was taken up by an energy-dependent system, by E. coli and S. cerevisiae (45). Furthermore an affinity series for monovalent cation uptake by S. cerevisiae has been given as $\text{K}^{+} > \text{Rb}^{+} > \text{Cs}^{+} > \text{Na}^{+} > \text{Li}^{+}$ (47).

MICROBIAL UPTAKE OF SPARINGLY SOLUBLE METALS

Iron is an essential metal for microorganisms. However, the metal exists predominantly as insoluble ferric hydroxide in aerobic environments. Microorganisms which inhabit these environments must be able to solubilize iron complexes for transport over the cell membrane. Such systems have been demonstrated to exist. They involve excretion of iron-chelating compounds, synthesized by the bacteria, and subsequent uptake of the ferric chelates by the organism. These iron-chelating compounds are termed siderochromes. They have been demonstrated to exist in many types of bacteria and yeast (48).

RECOVERY OF METAL IONS FROM SOLUTION VIA MICROBIAL ACTIVITY

Many heavy metals can be efficiently removed from water solutions as metal sulphides upon generation of H_2S by sulphate-reducing bacteria. Application of bacterial H_2S formation for metal recovery has been studied (49). A mixed culture of sulphate-reducing bacteria proved to be efficient in recovering and concentrating copper and zinc from a bacterial leaching solution on a laboratory scale. Growth of the sulphate-reducing bacteria and metal recovery demands a continuous culture system in which the metal ions are removed as sulphides immediately upon entering the fermentor.

DIFFERENT MECHANISMS IN MICROORGANISMS TO WITHSTAND INCREASING LEVELS OF TOXIC METALS

Microorganisms exhibit different abilities in changing their chemical physiology in order to compensate for alterations in their environment. However, the composition of the external media greatly influences the toxic effect of heavy metals present. For example, the toxicity of cadmium towards different bacteria and fungi was reduced when the growth media was supplemented with clay minerals (50).

The toxicity of Cd^{2+} to bacteria and fungi appeared to be pH dependent as the growth inhibition was potentiated in alkaline solutions probably due to the formation of $CdOH^+$ which was more toxic than Cd^{2+} (51). Other factors, such as the presence of amino acids, water hardness, phosphate and incubation temperature can affect the toxicity of heavy metals. Even agar growth media can neutralize the toxic effect of metals on bacteria (52). The neutralizing effect seemed to be a function of the soluble compounds of the media and not of the agar itself.

Much work has been devoted to mercury and its interaction with microorganisms. Resistance to mercury is always of an inducible nature. No examples of constitutive resistance to organic or inorganic mercurial compounds have been reported. Thus both resistant and sensitive cells exhibit cessation of growth upon exposure to mercury. Resistant cells recover growth after a delay of minutes or hours. A well documented pathway of detoxification of mercury is reduction of the toxic Hg^{2+} to metallic mercury which can be volatilized. Methylation of mercury can be performed by microorganisms but

this process appears to be performed by a genetic system separate from the mercuric reductase system. Methylated mercury accumulates in living organisms and is thus transferred to humans through the food chain.

Resistance to heavy metals in bacteria is usually due to genes carried on plasmids. It has been demonstrated that reduced cadmium uptake via an energy-dependent Mn^{2+} transport system in Staphylococcus aureus was due to the presence of a plasmid containing a gene for Cd^{2+} resistance (53). Furthermore, Cd^{2+} resistance in S. aureus seems to be due to an energy-dependent efflux system for cadmium thus preventing internal accumulation of Cd^{2+} (54). Uptake of cadmium by cells not harbouring the cadmium resistance plasmid is irreversible and leads to permanent inhibition of respiration.

Certain bacteria which are resistant to elevated levels of heavy metals appear to be able to compartmentalize the toxic ions within the cell. When mammalian cells are exposed to copper, cadmium or zinc, most of the ions are bound to specific inducible proteins. These sulphur-rich proteins have also been isolated from plants and yeast. Some evidence exists that these proteins, with a high affinity for toxic metals, are also synthesized by pro-caryotic organisms.

ADSORPTION OF HEAVY METALS TO MICROORGANISMS

Progressive attempts in using microbial cells as biosorbents for heavy metals and radioactive compounds from the nuclear fuel cycle have indicated that this alternative is advantageous when compared with current techniques for the detoxification of metal laden industrial effluents.

In striking opposition to the energy- and temperature-dependent uptake of metal cations, passive coordination of cations to negatively charged sites at the surface of the organism is rapid, reversible and independent of the energy supply.

The sorption of metal cations by microbial cell walls is an interesting item since all soluble material, such as metal cations, must contact and pass through the cell wall before reaching the plasma membrane. As surfaces of cells have a high anionic charge density it is to be expected that both cell walls and cell membranes interact with and bind cations.

In a study (55) regarding uptake and retention of 18 metals by cell wall fragments prepared from Bacillus subtilis it was indicated that specific mechanisms and binding sites exist in the bacterial cell wall. Substantial amounts of iron, magnesium, copper, sodium and potassium were adsorbed by the cell walls when exposed to 5 mM metal solution for 10 min. As much as 200 mg of Fe^{3+} and Mg^{2+} , 190 mg Cu^{2+} , 76 mg K^{+} and 62 mg Na^{+} could be bound by each gram (dry weight) of cell walls. Retention of metals by cross-linked cell walls was estimated in a column. Twelve metal solutions were passed through the column. Iron, magnesium, calcium and nickel were most strongly retarded. The total amount of metal bound by the cell wall fragments was 0.44 g per g (dry weight) walls.

Possible binding sites available in cell walls are the free carboxyl groups of the peptidoglycan and to a lesser extent the sugar hydroxyl groups and the amide groups of the peptide chains. In addition to the peptidoglycan, bacterial cell walls contain other polymers. The teichoic acids, present in the cell walls of Gram-positive bacteria, contain phosphodiester groups which can chelate metal cations.

The cell envelopes of Gram-negative bacteria provide a more complex system, both with regard to structure and chemistry, than the cell walls of Gram-positive bacteria. In some studies the metal adsorption to cell envelopes of Gram-negative bacteria has been investigated. Envelopes from E. coli (56) cannot bind as much iron, magnesium, copper, sodium and potassium as cell walls from B. subtilis. Of 32 metals screened, the transition elements Os and Hf were bound in the largest amounts (> 0.9 mmol/g dry weight). The envelopes were exposed in 5 mM metal solutions for 10 min.

Accumulation of uranium by microorganisms has been of special interest mainly due to the expected uranium shortage in the future. However, recovery of uranium from aqueous systems firstly requires very effective uptake processes, due to the low concentration of uranium in natural aqueous environments and secondly the adsorbing agent must bind uranium selectively as interfering cations will always be present.

Biosorption of radionuclides by non-living fungal biomass of Rhizopus arrhizus has shown promising results. Considerable amounts of thorium (170 mg/g dry weight) (58) and uranium (180 mg/g dry weight) (57) can be taken up from aqueous solutions. Processes concerning adsorption of uranium and

thorium by R. arrhizus have been proposed. For uranium the adsorption is thought to occur in a three step process. First uranium is complexed by the chitin molecules in the cell wall. Second, initially complexed uranium may act as nucleation sites for further deposition of uranium in the fungal cell wall. The third process involves hydrolysis of the uranium-chitin complex and the precipitation of the hydrolysis product in the cell wall. The now free amine nitrogen of the chitin may again complex uranium. The adsorption of thorium is considered to consist of only two processes. The first process involves complexation of thorium to the amine nitrogen of chitin where it remains fixed. In the second process thorium is adsorbed by the outer section of the cell wall. Furthermore, the results in these reports indicate that process application is suitable. Equilibrium is reached within 60 seconds of contact. Other cations present in the solution to be treated can however interfere with uranium uptake and thus render the process more difficult.

Accumulation of uranium by S. cerevisiae and P. aeruginosa (59) has shown that the metal uptake mechanism exhibited by these organisms differs considerably. The yeast adsorbs uranium on the cell surface or to polymers associated with the cell wall. The uptake is influenced by equilibrium pH, temperature and competing cations. The bacteria, on the other hand, show rapid absorption of uranium in a process unaffected by environmental parameters. Another interesting observation in this report is that only part of the cell populations maintained visible deposits of uranium.

In a series of reports regarding accumulation of heavy metal elements in biological systems the ability of different algae, bacteria, yeast and fungi to bind heavy metals have been studied. Exposure of Chlorella regularis to individual metal ions and to solutions containing equal concentrations of different heavy metal ions, revealed some interesting findings. The selectivity of heavy metal binding is about the same for living and heat-killed algal cells (60). However, the amount of metal ions taken up by living Chlorella cells is about half of the amount bound by heat-killed cells (61, 62). Metal uptake studies with chemically treated Chlorella showed that the crude protein content of the cells was approximately proportional to the amount of uranium adsorbed by the cells. It was furthermore concluded that the uptake of uranyl ions was scarcely influenced by the presence of copper and cadmium. Cadmium uptake, on the contrary, was negatively affected by the presence of uranium and copper. Screening of microorganisms for their ability to accumulate uranium (63) revealed that two organisms, Actinomyces

levoris and Streptomyces viridochromogenes were superior to the others tested. These organisms accumulated uranium in a rapid process unaffected by temperature and metabolism. Large amounts of uranium could be adsorbed on the surface of the organisms (0.25 g/g dry weight) and the metal was easily released from the organisms by washing the cells with EDTA.

Removal of metal ions from water by using polysaccharide producing bacteria as an adsorbing agent has shown interesting results. It was demonstrated that the cell floc matrix, produced by Zoogloea ramigera, accumulated 13-34 % of its own weight as metal ions. The cations tested were: iron, cobalt, copper and nickel (14). It was furthermore observed (64) that potassium was released from the floc matrix into the surrounding solution as other cations were adsorbed. Since the cell floc matrix was washed with distilled water before exposure to the solution containing cations, the authors suggested that potassium ions were adsorbed to the bacterial cells and the polymer during growth and then released by an ion exchange mechanism upon exposure to other cations. Eight species of floc-forming bacteria were isolated from sewage water and all of them were able to adsorb metal ions. Among these bacteria, Z. ramigera 115 showed superior ability to adsorb metal ions from acid mine water (64).

Steps towards a process using metal adsorbing cells immobilized on a carrier have recently been taken (65). Both algal and bacterial cells were immobilized by different methods. The cells immobilized with polyacrylamide had superior performance properties in comparison to the other methods. Uranium adsorption by the immobilized cells was performed in a column. High adsorption ability was demonstrated and the recovered uranium could be released from the cells with Na_2CO_3 . This technique or variations of it has potential for future demands. However, further development is needed regarding process design. The adsorbing agent must fulfill demands concerning metal binding capacity and selectivity. Cheap methods to immobilize microorganisms must also be found in order to reduce process costs.

MICROBIAL FLOCCULATION AND HOW IT AFFECTS METAL RECOVERY

Microbial flocculation is a process of bringing together freely suspended microorganisms to form aggregates or flocs with a size many orders of magnitude greater than that of a single microbial cell. A floc can be consi-

dered as a three-dimensional network resulting from the interactions between polymer and microbial cells.

Factors influencing microbial flocculation are of microbiological and environmental nature. Growth media composition and physiological age of the culture are microbiological factors which strongly affects flocculation.

Temperature, ionic properties of the medium, pH and presence of chelating agents are some environmental factors that govern floc formation. Experiments have shown that pH changes have a profound effect on bacterial aggregation. The clumping of Corynebacterium xerosis (66) was most intense when the bacteria were suspended in a solution at pH 3. Flocculation of S. cerevisiae (67) was low at pH 2 but the tendency to aggregate increased when pH increased and reached a maximum between pH 4.5 and 5.5. Addition of calcium improved floc formation. However in the presence of NaCl, higher concentration of CaCl_2 were needed to achieve maximum floc formation. Bacterial cells, which are negatively charged, can be flocculated by synthetic polymers (68). Negatively and positively charged as well as non-ionic polymers were found to be effective flocculating agents. However, using negatively charged or uncharged polymers necessitated the presence of multivalent cations.

C SUMMARY OF THE PAPERS

1 Production of extracellular polysaccharide by Zoogloea ramigera

In batch cultures of Zoogloea ramigera the maximum rate of exopolysaccharide synthesis occurred in a partly growth-linked process. The exopolysaccharide produced was attached to the cells as a capsule. The attached polysaccharide was released from the cell wall after 150 h of cultivation, which increased the viscosity of the fermentation broth. However, the polysaccharide synthesis was terminated within 72 h. Ultrasonication could be used to release capsular polysaccharide from the cell walls after synthesis had terminated but before the release phase was initiated. Feeding with glucose, nitrogen and yeast extract to a batch fermentation improved the polysaccharide production. The importance of nitrogen limitation for the polysaccharide production was demonstrated.

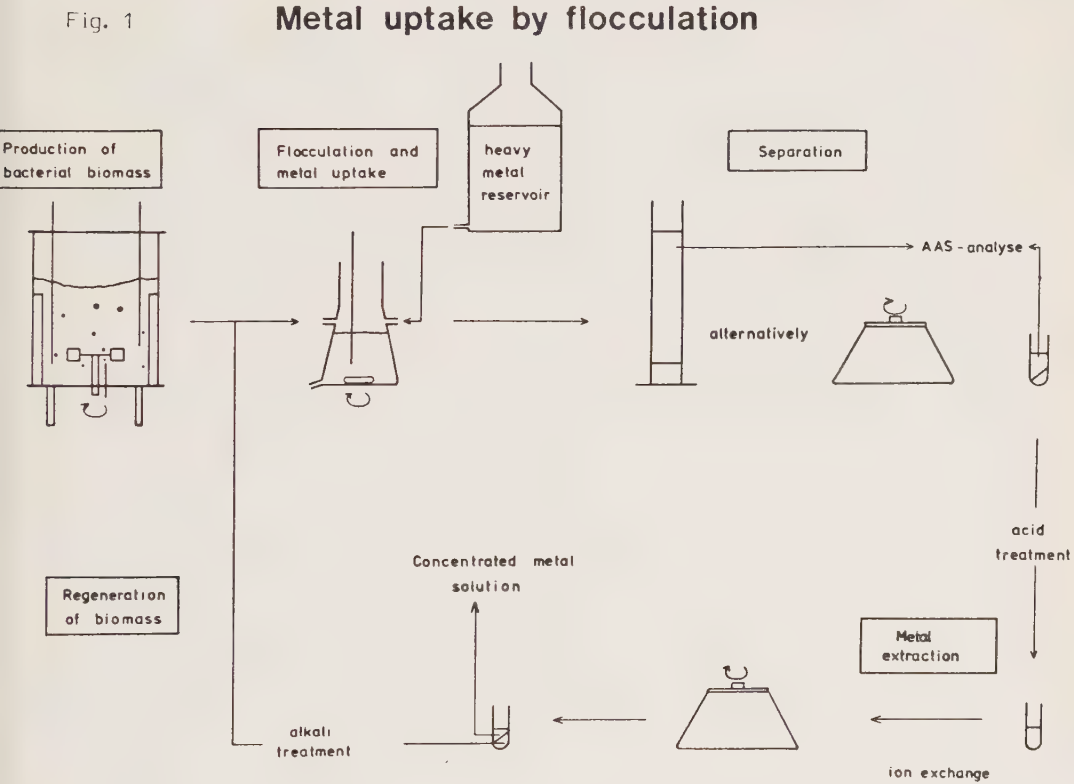
2 Toxicity of cadmium, cobalt, uranium and zinc to Zoogloea ramigera

Zoogloea ramigera, initially isolated from sewage sludge, is a common organism in sewage-treatment plants mainly due to its ability to degrade a diversity of carbon sources. The growth of the organism was strongly retarded in media supplemented with heavy metals. The length of the lag phase was proportional to the metal concentration when the organism was exposed to cadmium, cobalt and zinc. Upon exposure to uranyl ions the organism responded by showing an extended lag phase almost independent of the metal concentration used. The order of toxicity of the metals to the organism was $\text{Cd}^{2+} = \text{Zn}^{2+} > \text{Co}^{2+} > \text{UO}_2^{2+}$.

3 Accumulation of heavy metal ions by Zoogloea ramigera

A reversible process for metal accumulation is described (Fig. 1). The adsorbing agent was composed of Zoogloea ramigera cells and an acidic polysaccharide produced by the bacterium from glucose. The biomass was exposed to heavy metal ions, flocculation occurred at appropriate pH. The formed flocs, containing bacterial cells, polysaccharide and bound metal, were easily separated from the solution. The bound metals could be released from the floc matrix by adding acid. The biomass could be reused for metal accu-

mulation. The metal uptake was studied with two different methods; either by spectrophotometric measurements on the metal solutions after flocculation or by potentiometric measurements with amalgam electrodes in order to follow the entire complex formation.



4 Selective accumulation of heavy metal ions by *Zoogloea ramigera*

Treatment of solutions containing a mixture of cadmium, copper and uranium were performed with cells and exopolysaccharide produced by the bacterium *Zoogloea ramigera*. Exposure of biomass to the metal cations resulted in the formation of aggregated particles or flocs. These aggregates could easily be separated from the metal solution. The results indicated that selective accumulation of metal ions can be performed by adjusting pH to appropriate

levels. Trivalent aluminium was more effective than divalent magnesium and monovalent potassium in preventing divalent copper from being adsorbed to the biomass.

5 Heavy metal adsorption by Zoogloea ramigera compared to other adsorbing agents

The uptake of copper by Zoogloea ramigera, Pseudomonas denitrificans, polyacrylic acid and three different forms of carboxymethylcellulose was studied. Zoogloea ramigera was found to be the most effective adsorbing agent. The selective adsorption of copper and cadmium exhibited by Zoogloea ramigera was not shown by polyacrylic acid. The metal uptake was measured either by using atomic absorption spectrophotometric measurements or by potentiometric methods.

6 Development of a continuous process for metal accumulation by Zoogloea ramigera

A process for continuous recovery of copper from solution is described. The adsorption of copper to biomass produced by Zoogloea ramigera was studied with regard to retention time, biomass concentration and acidity. The results indicated that the uptake of metal is a rapid process taking place within minutes. The adsorption of copper to biomass is most efficient at low biomass concentrations. At high biomass concentrations, the total amount of copper removed from solution is high, but the specific amount of metal adsorbed to biomass is low.

7 Tillverkning och användning av biopolymer

Improved methods for the production of polysaccharide by Zoogloea ramigera is described. The production of polysaccharide is completed within 50 h in batch cultures with and without feeding. This is accomplished by addition of ferric ions during the cell growth phase. Optimal conditions for polysaccharide production in cultivations with feeding are obtained by starting the fermentation with a medium having a C/N ratio around 19. The glucose concentration is 25 g/l. When the initial amount of glucose is consumed feeding

of glucose is initiated. The addition of glucose can be controlled automatically. Feeding control is based on measurements of concentrations of CO_2 in the outlet gas. Several potential biotechnological applications for the biomass produced by Zoogloea ramigera are mentioned.

D DISCUSSION

EXOPOLYSACCHARIDE PRODUCTION

Bacterial exopolysaccharides are generally secreted into the surrounding medium after synthesis. Even moderate concentrations of polysaccharide in the cultivation medium have an adverse impact on mass transfer. As microbial synthesis of exopolysaccharides, with some exceptions, is dependent on progressive energy metabolism, sufficient oxygen must be transferred to the cells during the cultivation. Thus, to maintain reasonable levels of dissolved oxygen in highly viscous fermentation broths necessitates a high energy input. Slime producers, such as Xanthomonas and Pseudomonas, have a high demand for oxygen throughout the fermentation process. This includes both the initial cell growth phase, which is easily aerated, and the polysaccharide production phase, which is hard to oxygenate due to the reduction of the oxygen transfer coefficient induced by the viscous fermentation broth.

Production of exopolysaccharide by Zoogloea ramigera exhibits a more favourable pattern concerning the conditions governing oxygen transfer. The polysaccharides formed during the synthesis phase are anchored to the surfaces of the cells as voluminous capsules. As long as the capsular material is attached to the cells the viscosity of the broth is low. When the polysaccharides are detached from the cells the viscosity of the broth increases sharply. The synthesis phase and the release phase are quite distinct separated from each other.

Consequently, high oxygen transfer rates can be maintained during the synthesis phase. During the release of the polysaccharide from the cells, the demand for oxygen is low, which is favourable since it is difficult to maintain the oxygen transfer rate at a high level when the viscosity of the broth increases.

Attempts to increase the final concentration of polysaccharide in the fermentation broth were performed by feeding of fresh medium into the fermentor. As nitrogen limitation is important for polysaccharide production we assumed that synthesis of additional exopolysaccharide could be achieved by adding glucose. However, by supplying glucose the final concentration of polysaccharide did not increase. A possible explanation for this behaviour could be that the limited cell mass present was not able to convert more

glucose than the amount added at the start of the fermentation. But when the feeding solution contained glucose, NH_4Cl and yeast extract rapid conversion of glucose into polysaccharide was observed. Furthermore, the cell mass produced corresponded to the amount of nitrogen source added. These results indicate that cell growth can be initiated after the onset of polysaccharide synthesis and the concomitant capsule formation. Furthermore, the amount of exopolysaccharide formed is proportional to the cell mass and rapid conversion of glucose into exopolysaccharide is observed when nitrogen source combined with glucose is added (Paper I).

The addition of iron to the growth medium stimulated exopolysaccharide production. If addition of iron was performed from the beginning of the cultivation, the bacterial growth phase was terminated within 10 h (Paper VII). Glucose concentration reached zero after 50 h of cultivation. It is reasonable to assume that the shorter cultivation time attained is due to the addition of ferric ions. Furthermore, the formation of capsules is not influenced by the administration of ferric ions. Thus, the favourable conditions governing oxygen transfer during the production phase are consequently not affected.

RETRIEVAL OF METAL IONS

The phenomenon of adsorption of metals by microbial biomass probably reached its peak value at the time when living organisms started to colonize the earth. Recent geological investigations consider it possible that much of the soluble metal in our environment has been chelated by organic molecules which have then settled to form natural sediments (69). As a matter of fact, traces of microbial cells and high concentrations of associated metals have been localized in sediments from ancient geological formations (70). The association of microorganisms with manganese nodules in both marine (71) and limnological (72) systems is evident. The aim of most studies has been to recover economically valuable metals such as copper and uranium from polluted process water or from sea water. Zoogloea ramigera, the organism used in the present study is sensitive to increasing concentrations of cadmium, cobalt, uranium and zinc (Paper II). The sensitivity observed and the short exposure time needed to saturate the retrieving agent with copper or cadmium (Paper III and VI) indicate that the uptake of metal ions by biomass produced by Zoogloea ramigera is of a passive nature. Further-

more, it was observed that uptake of metal ions occurred in systems containing no glucose. Uptake of metal ions by a passive process requires no additional energy source.

A system for the retrieval of heavy metals using microbial cells or other biological reagents as adsorbing agents must fulfill certain basic demands. Thus, a short contact time is advantageous for process application. As mentioned above the metal uptake phase is completed within minutes. Active uptake of metal ions by living organisms is usually performed at lower rates than passive adsorption. On the other hand active accumulation of metals essential for microbial growth is usually carried out with greater selectivity than by metal retrieving systems based on passive adsorption. However, selective adsorption of heavy metal ions to biomass produced by Zoogloea ramigera has been demonstrated (Paper IV). From a solution containing cadmium, copper and uranium each metal could be recovered selectively. The retrieving system was by no means perfect as some copper and cadmium was removed during the uranium binding phase. However, it seems reasonable to assume that the system will be refined after further developments. Moderate concentrations of sodium and magnesium had no effect on the uptake of copper while aluminium interfered more strongly. Thus, if the aim of the process is the recycling of certain metal ions, trivalent cations such as aluminium must be removed in a separate process before more valuable metals are recovered from the solution. However, if the aim is only to reduce the concentration of some toxic but cheap metals, selective accumulation of the metal is not of primary importance.

It is evident that the adsorbing capacity of the biomass produced by Zoogloea ramigera is superior to the metal uptake capacity shown by some other agents (Paper V). Exposure of Pseudomonas denitrificans or charged carboxymethylcellulose to copper did not result in copper uptake at the level demonstrated by the mixture of microbial cells and charged polysaccharide produced by Zoogloea ramigera. Numerous studies have demonstrated that flocculation of bacterial cells is stimulated in the presence of polymers. As excellent flocculation occurs when biomass, consisting of Zoogloea ramigera and the polysaccharide synthesized by the organism, is exposed to heavy metals it seems that not only very favourable conditions for flocculation are obtained when a mixture of cells and polymer is exposed to metal ions but furthermore that the combination of cells and polysaccharide in the sample is essential for the efficient recovery of heavy metal ions.

A step towards field applications is demonstrated in Paper VI. The optimal conditions mentioned in this paper for the retrieval of the heavy metal in question have to be balanced against processing costs. If large volumes of water contaminated by a valuable metal are going to be treated one maybe has to sacrifice a certain percentage of the metal removal efficiency by using a lower concentration of biomass which will subsequently cut down the processing costs. However, as the adsorbing agent can be regenerated and reused final cost could be kept quite down.

E GENERAL CONCLUSIONS AND FUTURE PROSPECTS

EXOPOLYSACCHARIDE PRODUCTION

Microbial exopolysaccharides are widely distributed in nature. At the present time the phenomenon of microbial production of extracellular polysaccharides is of broad interest and concerns and presents exciting challenges in many areas of applied microbiology. Exopolysaccharides produced by bacteria have, in several cases been shown to have unique and interesting properties compared to existing polysaccharides. Although the market is currently limited to a few products it should be possible to produce new polysaccharides with physical and biological properties to form a complement to existing polysaccharides. However, further screening for potent polysaccharide producers is necessary. An interesting alternative could be the development of polymers with known characteristics to make new products with altered properties. The need for research within this field is obvious in order to improve existing processes for polysaccharide production and to unfold new techniques. Areas of special concern for future development could be; genetic improvement of exopolysaccharide producing strains in order to achieve higher yields and an increased production rate; control of parameters influencing polysaccharide composition; process control; product recovery. Progress within any of these areas would probably reduce production costs and thus improve the possibilities of commercialization.

METAL RECOVERY BY MICROORGANISMS

The outlook for biological enrichment of metals from water solutions seems to be bright. Since the first deliberate attempt was made to use biological materials as adsorbing agents for metal ions, considerable work has been done on the mechanisms and kinetics of metal accumulation by microbial cells. However, the maximum usefulness of microorganisms in metal accumulation has probably not yet been demonstrated. The main benefits to be sought in any application of microbials as metal-retrieving agents are greater selectivity and/or reduced costs when compared with existing chemical methods. Development of new biological processes for metal recovery should ideally aim at a process design which allows the recycling of adsorbing biomass. However, systems recovering valuable metals could possibly afford to give up the adsorbing agent. Furthermore, biological methods could not be

used in isolation when problems concerning metal pollution must be solved. The niche for biological methods must be identified and it seems realistic to assume that biological methods in the future will be used in combination with chemical and physical methods.

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H APPENDIX: Papers I-VII

Production of Extracellular Polysaccharide by *Zoogloea ramigera*

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In batch cultures of *Zoogloea ramigera* the maximum rate of exopolysaccharide synthesis occurred in a partly growth-linked process. The exopolysaccharide was attached to the cells as a capsule. The capsules were released from the cell walls after 150 h of cultivation, which caused the fermentation broth to be highly viscous. Ultrasonication could be used to release capsular polysaccharide from the microbial cell walls. Treatment performed after 48 to 66 h of cultivation revealed exopolysaccharide concentration and apparent viscosity values in accordance with values of untreated samples withdrawn after 161 h of cultivation. The yield coefficient of exopolysaccharide on the basis of consumed glucose was in the range of 55 to 60% for batch cultivations with an initial glucose concentration of 25 g liter⁻¹. An exopolysaccharide concentration of up to 38 g liter⁻¹ could be attained if glucose, nitrogen, and growth factors were fed into the batch culture. The oxygen consumption rate in batch fermentations reached 25 mmol of O₂ liter⁻¹ h⁻¹ during the exopolysaccharide synthesis phase and then decreased to values below 5 mmol of O₂ liter⁻¹ h⁻¹ during the release phase. The fermentation broth showed pseudoplastic flow behavior, and the polysaccharide was not degraded when growth had ceased.

Several bacteria are known to synthesize large amounts of extra-cellular polysaccharides, resulting in mucoid colonies on agar plates and extremely viscous broth during submerged cultivation. In many cases these polysaccharides have found technical applications, as for instance in the food and pharmaceutical industries (15). In most cases the production is oxygen dependent, and due to the high viscosity of the fermentation broth, oxygen transfer is one of the critical factors in microbial polysaccharide production (10). One well-known type of bacterium able to produce slime is *Zoogloea ramigera* (12). This bacterium is frequently found to accumulate in sewage plants (2). The exopolysaccharide formed by *Z. ramigera* strain 115 behaves like a polyelectrolyte and shows a strong affinity to metal ions (5), which makes it a potential agent for metal recovery.

This report is part of a general study on using microbial biomass as an adsorbent for heavy metal ions. Furthermore, *Z. ramigera* strain 115 has been shown to produce polysaccharide. If this property is further developed, it could be of future interest for industrial exploitation.

In this study we report on the relationship

between exopolysaccharide production and growth conditions and on the rheological properties of the fermentation broth.

MATERIALS AND METHODS

Organism. *Zoogloea ramigera* strain 115 (ATCC 25935) was obtained from the American Type Culture Collection, Rockville, Md.

Media. The organism was maintained on Trypticase soy agar (BBL 11043, BBL Microbiology Systems) with weekly transfer to fresh medium. The cultivation medium had the following composition (g/liter): glu-

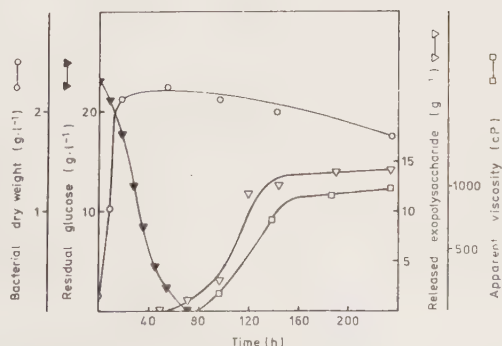


FIG. 1. Exopolysaccharide production by *Z. ramigera* in batch culture.

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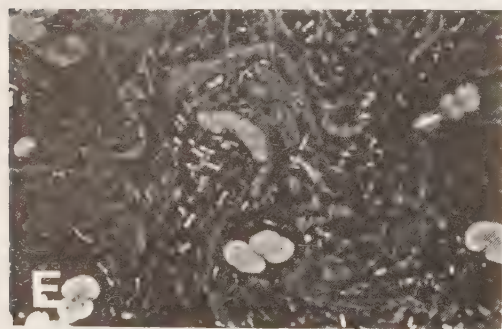
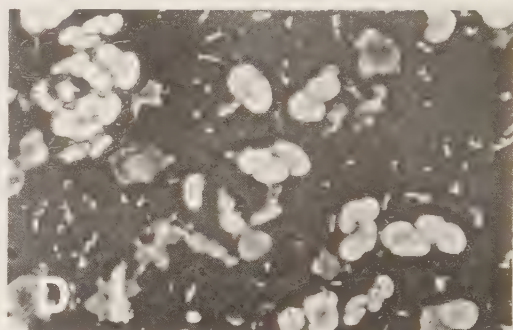
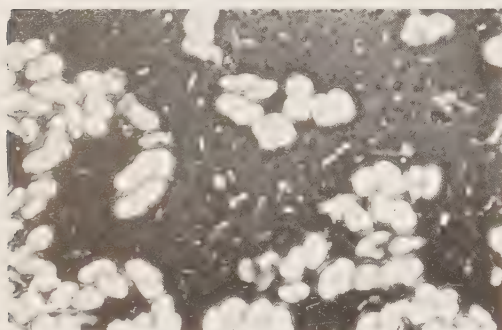
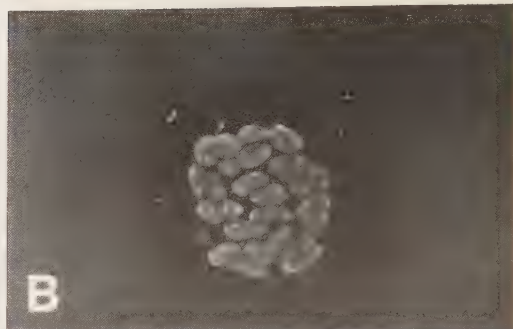


FIG. 2. Capsule of *Z. ramigera* demonstrated by dispersing the cells in 1% nigrosin. The photographs were taken with a phase-contrast microscope at different culture ages: (A) 24h, (B) 48 h, (C) 90 h, and (D) after 120 h of culture age. (E) At 66 h after sonication for 20 s.

cose, 25; K_2HPO_4 , 2; KH_2PO_4 , 1; NH_4Cl , 1; $MgSO_4 \cdot 7H_2O$, 0.2; yeast extract (Difco Laboratories), 0.01. Glucose and $MgSO_4 \cdot 7H_2O$ were autoclaved separately. The solution used for feeding contained (g/liter): glucose, 400; NH_4Cl , 16; yeast extract, 0.16. The components were autoclaved separately.

Culture conditions. Seed cultures were derived from a single colony isolate and grown for 24 h on a rotary shaker at 26°C in the medium defined above. These cultures were used for inoculation of a 3-liter working volume fermentation vessel (Chemoferm AB, Hågersten, Sweden) fitted with a six-blade open turbine and a condenser for the outlet air to reduce the evaporation of water during long-term experiments. The fermentation pH was maintained at 7.0 by titration with 1 M NaOH. The initial foaming was controlled by a mechanical foam breaker. Agitation was at 800 rpm, and the aeration was controlled at 0.5 VVM (volume of

air · volume of medium⁻¹ · min⁻¹), unless otherwise stated.

The C/N ratio of the medium was adjusted by altering the glucose concentration and keeping the concentration of nitrogen constant or by modifying the nitrogen concentration at constant glucose concentration.

Viscosity. Viscosity was measured with an STV Epprecht Rheometer (Contraves AG, Zürich) at 25°C. The apparent viscosities were determined at a fixed shear rate of 77.9 s⁻¹, unless otherwise stated. The consistency index (apparent viscosity at a shear rate of 1 s⁻¹) was determined by using a Rheomat-30 viscometer (Contraves AG).

Glucose determination. Glucose was determined by the hexokinase method using a Glucoquant kit (Boehringer Mannheim Corp.).

Oxygen measurement. Oxygen in the outlet gas was measured continuously with a paramagnetic oxygen analyzer (Leeds & Northrup, England).

Dissolved oxygen tension of the broth was measured with a galvanic oxygen electrode according to Johnson et al. (7).

TABLE 1. Influence of the C/N ratio on cell and polysaccharide production at a glucose concentration of 25 g liter⁻¹

C/N ratio	Polysaccharide concn (g/liter)	Bacterial dry wt (g/liter)	Yield coefficients (g/g)	
			Y_{PS}	$Y_{X/S}$
38	15.5	2.4	0.62	9.2
26	10.0	3.7	0.40	9.7
19	5.1	5.0	0.20	9.4

Cell-bound bacterial polysaccharide. Bacterial cells and capsular polysaccharide could not be thoroughly separated by centrifugation. Samples were first subjected to ultrasonication three times for 20 s each time at an amplitude of 10 μ m in an MSE Soniprep 150 (MSE Scientific Instruments, Crawley, United Kingdom). Centrifugation was then performed at 31,300 $\times g$ for 40 min to remove cells and cellular debris in a Beckman J2-21 centrifuge. When the sonicated sample became viscous, it was diluted with 3 volumes of distilled water before centrifugation to achieve good separation of the cells and polymer. KCl was added to the supernatant to a 1% (wt/vol) concentration. Precipitation of the polysaccharide was performed by adding 2 volumes of propanol. The resultant precipitate was filtered off by using a preweighed GF/A filter dish (Whatman Ltd., Maidenstone, England). The filter was dried at 105°C to a constant weight. The dry weight was determined gravimetrically.

Released exopolysaccharide. To determine the released portion of the exopolysaccharide, the withdrawn sample was diluted with 3 volumes of distilled water. Centrifugation was performed at 31,300 $\times g$ for 40 min. This sedimented the cells with and without capsules. The supernatant was decanted, and KCl was added to a 1% (wt/vol) concentration. Precipitation was performed by adding 2 volumes of propanol. The amount of released exopolysaccharide was then determined gravimetrically as described above.

Cell dry weight. The cell-bound polysaccharide was removed as described above, and the cells were resuspended in water to the initial volume. The dry weight was then measured gravimetrically using Sartorius membrane filters with a pore size of 0.2 μ m after desiccation at 105°C for 2 h.

DNase treatment. DNase treatment was performed to ensure that the increase in viscosity, obtained after sonication, was not due to liberation of DNA. Samples (40 ml), withdrawn from the fermentor at different culture ages, were sonicated three times for 20 s each time. The enzyme solution, prepared by dissolving 5,400 Kunitz units in 2 ml of 0.15 M NaCl, was added to the sonicated sample. The temperature was maintained at 25°C, and the pH was 7.0. Apparent viscosity was recorded continuously for 6 h after the addition of DNase (Sigma Chemical Co.). To demonstrate that hydrolysis of DNA occurred under these conditions, the increase in absorbance at 260 nm was measured.

A separate sample was supplied with magnesium (final concentration, 1 mM) to determine if sufficient magnesium was present to allow hydrolysis of DNA.

RESULTS

Production of exopolysaccharide in batch culture. The cell growth phase was finished within 20 h. However, the glucose consumption continued far beyond this time, as shown in Fig. 1. During the first 70 h of cultivation, microscopic analysis of the culture showed that the polysaccharide was attached to the microbial cells as capsules (Fig. 2). After about 90 h of culture, polysaccharide also occurred in the extracellular fluid, as measured by viscosity (Fig. 1). The final apparent viscosity value was in the range 800 to 1,000 cP. The consistency index of the broth was around 17,500 cP. The viscosity remained constant when the culture was held at 26°C for 100 h after the viscosity had reached its maximum value, thus indicating that no degradation of polysaccharide occurred. The yield of polysaccharide from glucose was around 60% on a weight basis.

The C/N ratio had a pronounced effect on the polysaccharide production. The final amount of exopolysaccharide was negatively affected if the C/N ratio was decreased below 38 at a fixed glucose concentration of 25 g/liter. Instead, the culture responded by producing more cells. The yield coefficient $Y_{X/S}$ was constantly around 9 irrespective of the C/N ratio (Table 1).

Results from gas analysis during batch fermentations show that the oxygen absorption rate reached a peak value of 25 mmol of O₂ liter⁻¹ h⁻¹ at 40 h of growth. During the viscosity-increasing phase the oxygen uptake never exceeded 5 mmol of O₂ liter⁻¹ h⁻¹. Krul (8), who studied the activity of *Z. ramigera* in flocs and suspension, showed that the potential oxygen demand of the cells occurred in the early growth phase of the cultures.

To verify that the microscopically observed capsules contained polysaccharide during the period when growth had ceased but glucose was still being consumed, samples were subjected to ultrasonication. This treatment resulted in the release of polysaccharide from the cells as could

TABLE 2. Effect of sonication on the apparent viscosity of the culture broth at different ages

Culture age (h)	Treatment	Resulting viscosity (cP)	Bacterial dry wt (g liter ⁻¹)	Released polymer concn (g liter ⁻¹)
48	None	5	2.0	0.5
48	Sonication	940	2.2	12.9
66	None	10	ND ^a	0.4
66	Sonication	2,670	2.1	13.2
161	None	880	2.3	14.0
161	Sonication	2,900	ND	ND

^a ND, Not determined.

TABLE 3. Polysaccharide production by *Z. ramigera* in fed batch system

Nutrients fed	Amt of glucose added (g liter ⁻¹)	Residual glucose concn (g liter ⁻¹)	Polysaccharide concn (g liter ⁻¹)	Polysaccharide yield from glucose used (%)	Bacterial dry wt (g liter ⁻¹)	Apparent viscosity (cP)
Glucose	42.6	16	12.5	47	2.5	520
Glucose plus NH ₄ Cl plus yeast extract ^a	53	0	32.8	62	5.0	2,340
Glucose plus NH ₄ Cl plus yeast extract	59.2	0	38	64	6.0	3,100

^a Concentration of dissolved oxygen above 15% of saturation throughout the cultivation.

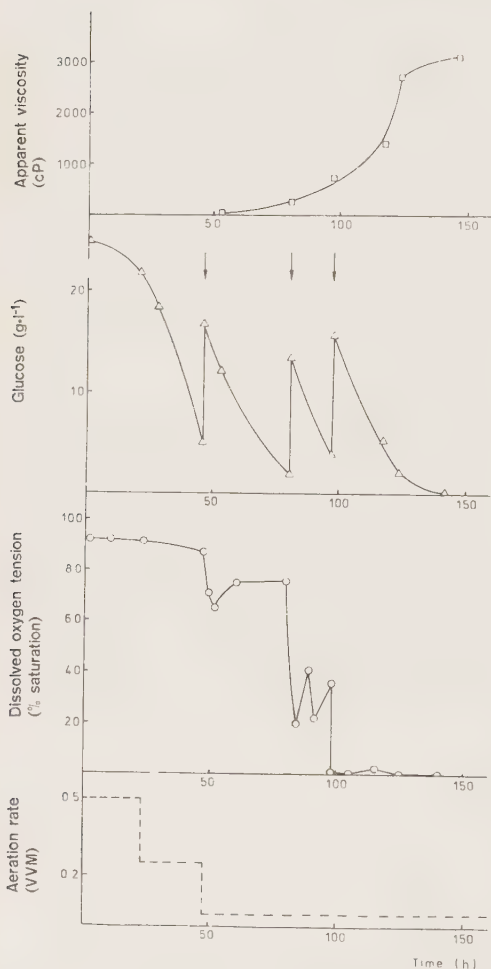


FIG. 3. Exopolysaccharide production by *Z. ramigera* in fed batch culture. Arrows indicate addition of feeding solution.

be observed microscopically (Fig. 2). The sonicated samples were also stained for light microscopy by using two dyes, namely, alcian blue 8GX and toluidine blue. Toluidine blue, which is a metachromatic dye, produced a red color

when exposed to the sonicated sample. Samples stained with alcian blue turned dark blue. Both reactions confirm that the sample contains polysaccharide. Furthermore, a steep increase in viscosity was obtained after sonication (Table 2). Cell mass and exopolysaccharide determinations performed on the sonicated samples were comparable to the results obtained on untreated samples after 161 h of culture. To check the presence of DNA in the medium after sonication, DNase was added to the sonicated sample. This treatment did not influence the viscosity. However, the presence of DNA was demonstrated, as we recorded an increased absorbance at 260 nm upon the addition of the enzyme. Addition of magnesium did not affect the hydrolysis.

Production of exopolysaccharide in fed batch culture. The first attempt to increase final polysaccharide concentration was performed by the feeding of glucose. Glucose was fed in two portions, the first after 34 h and the second after 71 h of cultivation. In both cases, glucose concentration in the fermentor increased by 9 g/liter. The results showed that no increase in final polysaccharide concentration was gained compared with batch cultivation. The final apparent viscosity was 500 cP (Table 3). In the following experiment the feeding solution also contained NH₄Cl and yeast extract. The C/N ratio of the feeding solution was 38. The solution was fed in two equal portions, each increasing the glucose concentration by 13 g/liter. Feeding was performed at 44 and 72 h of cultivation. The dissolved oxygen tension never decreased below 15% of air saturation during the polysaccharide production phase. The maximum polysaccharide concentration was reached within 100 h of growth, with a 63% yield on a glucose basis. The apparent viscosity was 2,340 cP, and the consistency index of the broth was 72,000 cP.

To evaluate the effects of oxygen limitation, another cultivation was performed in which the aeration rate was lowered from 0.5 VVM to 0.25 VVM at 23 h of growth and further decreased to 0.05 VVM at 47 h of growth. The feeding solution contained glucose, NH₄Cl, and yeast ex-

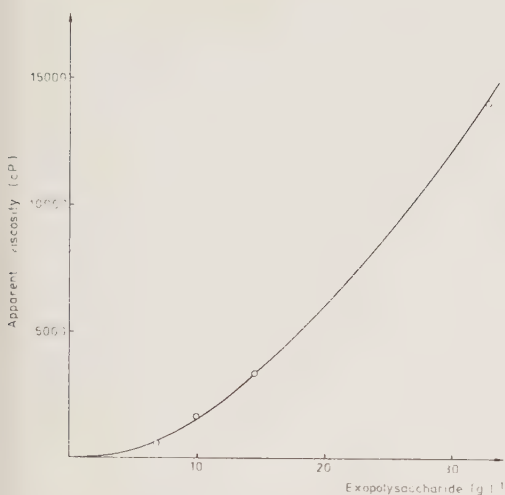


FIG. 4. Concentration of exopolysaccharide in fermentation broth versus apparent viscosity at a shear rate of 8 s^{-1} .

tract as described above. The solution was fed in three equal portions, each increasing the glucose concentration by 11 g/liter. Feeding was performed at 46, 81, and 97 h of growth. During the feeding experiment, glucose concentration in the fermentor never fell below 2 g/liter. After 145 h of cultivation, the apparent viscosity of the broth was 3,100 cP, and the consistency index of the broth reached 94,000 cP. Of the glucose supplied to the fermentor, 64% was converted into polysaccharide. The signal of the dissolved oxygen probe declined to values close to zero after the third addition of nutrients and remained at this low level throughout fermentation. Two factors caused this: first, increased oxygen consumption, and second, the fact that the probe is sensitive to viscosity (1) which reduces the signal of the electrode at increasing viscosity (Fig. 3 and Table 3).

Rheological properties of fermentation broth.

Figure 4 shows the apparent viscosity at 7.99 s^{-1} versus the concentration of exopolysaccharide in fermentation broth. The effect of temperature on the viscosity of fermentation broth containing 1.5% polysaccharide indicated an irreversible behavior when the temperature was successively raised from 25 to 90°C and back again. This phenomenon occurred only once and might indicate a thermal rearrangement of the polysaccharide during the first heat treatment that resulted in a higher viscosity at low temperature (Fig. 5). The viscosity of the cultivation broth, containing 1.5% polysaccharide, is essentially independent of pH (Fig. 6). Decreasing the pH

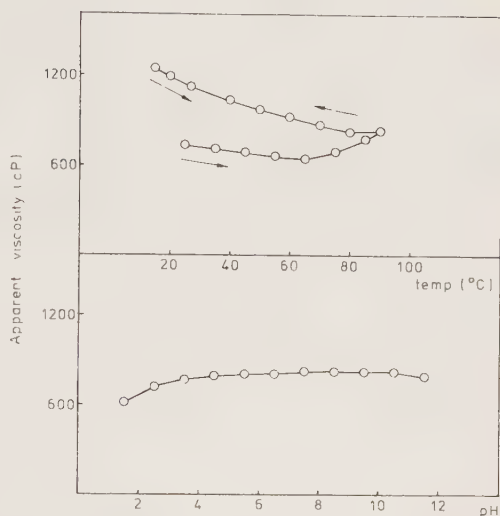


FIG. 5. Effects of temperature and pH on apparent viscosity of fermentation broth. Concentration of exopolysaccharide is 1.5%.

5). If pH is increased above 11, the polysaccharide forms a brittle gel which makes viscosity measurements impossible. Figure 6 shows the change in rheological behavior of the growing culture. As shown by the almost straight line corresponding to 81 h of cultivation, the broth was close to Newtonian at the beginning of the fermentation. The broth became pseudoplastic in its flow behavior at the beginning of the release period.

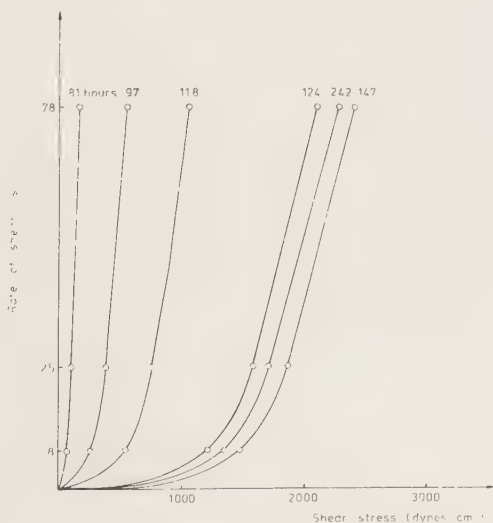


FIG. 6. Rheograms of the broth from a batch cul-

DISCUSSION

When growth ceased in the batch process (after 20 h), only 5 g of glucose per liter had been consumed, and about 2 g of biomass per liter had been formed. This gives a yield coefficient of biomass ($Y_{X/S}$) of about 0.4 (grams of cells per grams of glucose) which is in accordance with typical yield coefficients for aerobic growth on carbohydrates (9). In spite of the growth cessation, glucose continued to disappear from the broth and reached zero after 70 h without any concomitant increase of biomass or appearance of polysaccharide in the broth (Fig. 1). A possible explanation of this phenomenon would be that glucose is used in the stationary phase for synthesis of polysaccharides which are not excreted into the medium. The microphotos (Fig. 2) demonstrate the presence of large quantities of capsular material around the cells during the glucose consumption phase (0 to 70 h), and a further support for this hypothesis is the observation that sonication of these cells increases the viscosity of the medium (Table 2) and detaches the capsular material from the cells (Fig. 2).

The unexpected high viscosity of samples sonicated after 66 and 161 h raised the question whether cell disintegration and DNA release might have caused the increased viscosity during the treatment. However, treatment of the sonicated samples with DNase did not reduce the viscosity of the medium. Furthermore, the untreated 48-h sample contained no free polysaccharide, while the same sample after sonication showed a polysaccharide content of 12.9 g/liter (Table 2). These observations further support the hypothesis that polysaccharides are formed during the glucose consumption phase but stick to the cells as capsules till a later phase when they are at least partly released to the medium. To achieve a total release of polysaccharides from the cells, thorough sonication of the sample has to be performed (Fig. 2).

The data from the batch culture (Fig. 1) give the impression that it is glucose starvation that induces the release of polysaccharides to the medium. However, the results from the batch culture contradict this since the release of polysaccharides started at about the same time (60 to 70 h) in both types of cultures while in the fed batch culture the glucose concentration was high throughout the process.

The importance of nitrogen limitation for the polysaccharide production is shown in Table 1. The more nitrogen source in the initial medium, the less polysaccharides were produced. However, the cell amount produced was proportional to the initial concentration of nitrogen source as visualized by the constant yield coefficient $Y_{X/N}$. This is a common characteristic of exo-

polysaccharide production both by yeasts (4) and bacteria (6).

It was therefore expected that more polysaccharides could be produced by increasing the glucose supply by fed batch cultivation. However, more feeding with glucose did not improve the polysaccharide production. To increase the final product yield, the nitrogen source and yeast extract had to be supplemented (Table 3). With this technique, the culture reached viscosities so high that adequate mixing could not be achieved in an ordinary laboratory fermentor. Cadmus et al. (3) reported that 65% of the glucose consumed by *Xanthomonas campestris* was converted into xanthan gum. Parsons and Dugan (12) estimated that *Z. ramigera* converted approximately 34% of the carbon source supplied into exopolysaccharide. In our experiments, 60 to 64% of the glucose used was converted into exopolysaccharide.

Microbial synthesis of exopolysaccharides is dependent on phosphorylating reactions (14) and must therefore be dependent on energy metabolism which involves respiration in the case of aerobic organisms. Oxygenation of polysaccharide processes as in xanthan fermentation is a major technical problem because of the severe reduction of the oxygen transfer coefficient ($K_L a$) caused by the highly viscous broth. However, unlike *X. campestris* (13), *Pseudomonas aeruginosa* (11), and *Pseudomonas* NCIB 11264 (16) which formed and secreted polymer throughout the fermentation, *Z. ramigera* in our study produced polysaccharides which attached to the cell walls. This tendency of the polysaccharide to attach to the cells and not make the broth viscous during the synthesis phase allows high oxygen transfer rates to be maintained during the high oxygen demand period. Furthermore, very low oxygen consumption was observed during the period of polysaccharide release when the oxygen transfer rate cannot be raised to reasonable values without very high energy inputs.

ACKNOWLEDGMENTS

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TOXICITY OF CADMIUM, COBALT, URANIUM AND ZINC TO *ZOOGLOEA RAMIGERA*

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Abstract—The toxic effects of cadmium, cobalt, uranium and zinc to the bacterium *Zoogloea ramigera* have been studied. This organism, which is abundant in sewage treatment plants, responded to media supplemented with heavy metals by showing a prolonged lag phase and by decreasing its growth rate. The length of the lag phase was proportional to the metal concentration when *Z. ramigera* was exposed to cadmium, cobalt and zinc. When exposed to uranium (uranyl ions) the organism altered its growth behavior by showing an extended lag phase almost independent of the metal concentration. The order of toxicity of the metals to *Z. ramigera* was $\text{Cd}^{2+} = \text{Zn}^{2+} > \text{Co}^{2+} > \text{UO}_2^{2+}$.

Key words—toxicity, adaptation, resistance, bacteria, waste-water treatment, cadmium, cobalt, zinc, uranium, *Zoogloea ramigera*.

INTRODUCTION

Heavy metals emitted by industrial and domestic activities into the environment in many cases influence the activity of the microbial population. In the natural environment, the effects of heavy metals to a large extent depend on the chemical composition of the recipient into which the metals are deposited.

In accordance with this, laboratory studies concerning effects of heavy metals on microorganisms have shown that the composition of the culture medium greatly influences the apparent toxicity of metals to microorganisms (Babich & Stotzky, 1974, 1978).

The polysaccharide producing bacterium, *Zoogloea ramigera*, initially isolated from sewage sludge, is a common aerobic organism in sewage-treatment plants mainly due to its ability to degrade a wide spectrum of carbon sources (Dugan, 1975). Extensive amounts of polysaccharide can be produced by the organism when cultivated on appropriate media (Norberg & Enfors, 1982). The organism has been used for metal uptake experiments (Friedman & Dugan, 1968).

An important mechanism for reduction of the toxic effects of the heavy metals is their interaction and adsorption to microbial surfaces in the presence of excreted polymers. These charged polymers interact with the pollutants in activated sludge and have been shown to complex heavy metals (Brown & Lester, 1979).

This report is part of a study on microbial accumulation and concentration of heavy metals in effluents from mine and metal plating industries, using polysaccharide from *Z. ramigera* as adsorbent. The objectives of this first report were to determine the ability of the organism to withstand increasing concentrations of Cd^{2+} , Co^{2+} , UO_2^{2+} and Zn^{2+} . Of the

metals tested, cobalt and zinc are considered toxic but essential, while cadmium and uranium are toxic and to date without metabolic function.

MATERIALS AND METHODS

The organism used in this study was *Zoogloea ramigera* strain 115 (ATCC 25935). The bacterium was obtained on Trypticase Soy Agar (BBL 11043) slants.

The cultivation medium contained (g l^{-1}): glucose 20; KCl 2.3; NH_4Cl 1; $\text{C}_3\text{H}_7\text{Na}_2\text{O}_6\text{P} \times 5\text{H}_2\text{O}$ 1; $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ 0.2 and yeast extract (Difco) 0.01. By adding alkali, pH of the medium was adjusted to 7.0. Fifty ml of the above medium was inoculated with a loop of organisms from the agar plate. The inoculum was cultivated on a rotary shaker (200 rpm) at 26 °C for 24 h. This bacterial suspension was used for inoculating the test tubes (15 × 160 mm) containing 5 ml of the cultivation medium described above supplemented with Cd^{2+} , Co^{2+} , Zn^{2+} and UO_2^{2+} . The final metal concentrations were 0, 1, 5, 10, 50, 100, 500 and 1000 ppm for Co^{2+} , Zn^{2+} and UO_2^{2+} . Cd^{2+} was added to give final concentrations of 0, 1, 3, 5, 10, 25, 50 and 100 ppm. The metal salts used were CdCl_2 , CoCl_2 , $\text{UO}_2(\text{NO}_3)_2 \times 6\text{H}_2\text{O}$ and ZnCl_2 . All chemicals were of analytical grade. Each concentration was run in triplicate. The inoculum size was 0.1 ml, incubation was performed at 24 °C and aeration was maintained by shaking at 200 rpm. This shaking frequency supplied the culture with enough oxygen to attain logarithmic growth. Growth was measured by optical density with a Turner spectrophotometer at 620 nm. A calibration between turbidity and dry weight of biomass gave a linear relationship, where a turbidity of 1.0 corresponds to a dry weight of 2.2 g l^{-1} . Using total or viable count as a measure of growth was not applicable due to flocculent growth. Measurement of pH was performed at the beginning and at the end of all experiments.

According to Baes & Mesmer (1976), formation of hydroxylated species of Cd^{2+} , Co^{2+} and Zn^{2+} are avoided within the pH region 6–7. Uranyl ion, UO_2^{2+} , hydrolysis begins at about pH 3. The principal hydrolysis products in acidic solutions are $\text{UO}_2(\text{OH})^+$ and $(\text{UO}_2)_2(\text{OH})_2^{2+}$. $(\text{UO}_2)_3(\text{OH})_4^+$ is the dominant species at pH higher than about 5.

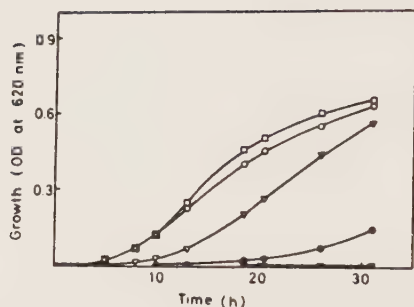


Fig. 1. Effect of cadmium on the growth of *Z. ramigera*. Symbols: $\square$, control cells; $\circ$, 1 ppm Cd; ∇ , 3 ppm Cd; $\bullet$, 5 ppm Cd; $\blacktriangledown$, 10 ppm Cd.

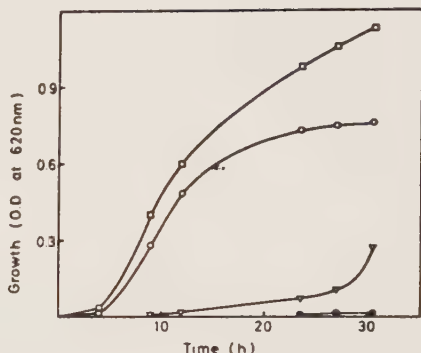


Fig. 3. Effect of zinc on the growth of *Z. ramigera*. Symbols: $\square$, control cells; $\circ$, 1 ppm Zn; ∇ , 5 ppm Zn; $\bullet$, 10 ppm Zn.

RESULTS

Increasing concentrations of cadmium in the growth medium resulted in a prolonged lag phase and decreased specific growth rate of *Z. ramigera* (Fig. 1). The elongation of lag phase due to cadmium ions is linearly proportional to the concentration of cadmium. The toxic level of Cd for *Z. ramigera* was 10 ppm. The tubes were inoculated at zero time and the exposure continued for 32 h.

When cobalt was added to the growth medium the culture responded with increased lag phase at a cobalt concentration of 10 ppm. At a concentration of 50 ppm, cobalt totally inhibited growth of *Z. ramigera* during the experiment which lasted for 32 h (Fig. 2). The origin on Fig. 2 is the time of inoculation.

Zinc had a severe effect on growth of *Z. ramigera* (Fig. 3). Increased lag phase was demonstrated already at a concentration of 1 and 5 ppm, 26 h elapsed before logarithmic growth started. The toxic level of Zn for *Z. ramigera* was 10 ppm during this experiment which lasted for 32 h.

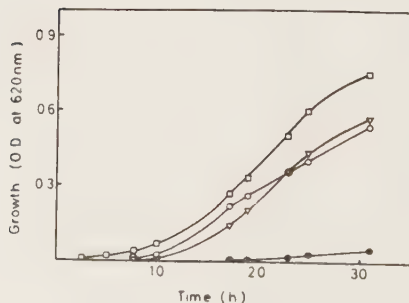


Fig. 2. Effect of cobalt on the growth of *Z. ramigera*. Symbols: $\square$, control cells; $\circ$, 1 ppm Co; ∇ , 5 ppm Co; $\bullet$, 10 ppm Co.

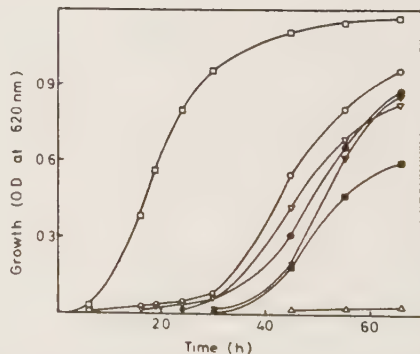


Fig. 4. Effect of uranium on the growth of *Z. ramigera*. Symbols: $\square$, control cells; $\circ$, 1 ppm U; ∇ , 5 ppm U; $\bullet$, 10 ppm U; $\blacktriangledown$, 50 ppm U; $\blacksquare$, 100 ppm U; $\triangle$, 500 ppm U.

Altering the size of inoculum (0.05 or 0.1 ml) did not significantly change the length of the lag phase or the critical concentration of metal which inhibited growth.

DISCUSSION

Microorganisms exhibit different sensitivity towards heavy metals. Organisms which are resistant to inorganic ions have usually acquired this ability through adaptation (to toxic quantities) during long-term exposure. Abiotic factors greatly affect the toxicity of heavy metals. According to Babich & Stotzky (1979), inorganic phosphate and soluble organic like yeast extract reduce toxicity of heavy metals. When studying anaerobically digested sludge, Gould & Genetelli (1977) found that the equilibration pH affected the complexation of metals. Lowering of pH resulted in higher soluble metal concentrations. Adsorption of heavy metals to activated sludge was also sensitive to environmental conditions (Cheng *et al.*, 1975; Neufeld & Herman, 1975).

In our experiment, complexation of the heavy metals tested was minimized by addition of glycerophosphate instead of inorganic phosphate and by keeping the amount of yeast-extract added at a low level. *Z. ramigera* responded immediately to growth medium containing heavy metals. Elongation of the lag phase was the most predominant effect. This behavior is also shown by *Escherichia coli* when exposed to cobalt (Fujiwara *et al.*, 1977). Mitra *et al.* (1975) demonstrated that *E. coli* had an extended lag phase when exposed to cadmium.

The specific growth rate of *Z. ramigera* declined upon exposure to media supplemented with Cd, Zn and Co. This behavior is in accordance to results obtained with *Klebsiella aerogenes* when exposed to Cd and Zn (Pikett & Dean, 1976) and furthermore to *Pullularia pullulans* when cultured in the presence of Cu (Gadd & Griffiths, 1978). Mitra *et al.* (1975), described that when using accommodated *E. coli* B, the doubling time was markedly increased upon exposure to higher concentrations of Cd.

The reduction in the growth rate noted suggests that *Z. ramigera* responds to changes in the environment by altering its cellular metabolism.

When exposed to uranium, *Z. ramigera* responded in a different manner than when exposed to the other metals. The lag phase was not correlated to the concentration of uranium in the growth media which had been noted for Cd, Co and Zn. Samples supplemented with 1–100 ppm U had a lag phase of equal length.

Literature data on uranium toxicity is scarce. Tuovinen & Kelly (1974) showed that the addition of 5–100 mM uranium to growing cultures of *Thiobacillus ferrooxidans* caused rapid loss of viability.

Our experiment shows that *Z. ramigera* is more sensitive to uranium. Inhibition of growth occurs at 1000 ppm of this metal. However, these two experiments cannot be compared because of the hydroxyl-

ated uranium species present in our experiment, but not in the experiment on *T. ferrooxidans*. These species can potentiate or reduce the toxicity of uranium towards *Z. ramigera*. Babich & Stotzky (1978) demonstrate that the hydroxylated species $\text{Cd}(\text{OH})^+$ was more toxic to bacteria than divalent Cd^{2+} .

On the other hand Babich & Stotzky (1979) report that hydroxylated species $\text{Pb}(\text{OH})^+$ are less toxic than divalent Pb^{2+} to fungi. This observation can explain the result obtained in our study. Cadmium, cobalt and zinc exist as highly toxic divalent cations at pH 6–7 while uranium exists as less toxic hydroxylated species at the same pH. Equilibrium pH of the growth medium strongly influence not only the toxicity of heavy metals to microorganisms but perhaps more the accessibility of the heavy metal ions to the microorganism. The metal must gain access to the microbial cell in order to influence the metabolism of the cell. It seems reasonable to assume that nonhydroxylated species more easily than hydroxylated species can gain access to the microbial cell.

Walker & Houston (1981), demonstrated that *Pseudomonas aeruginosa* and *Aeromonas* sp. adapted to very high levels of cadmium upon prolonged incubation. In our experiments, *Z. ramigera* showed an adaptation to increasing concentrations of metals within the time period studied. *Z. ramigera* is one of the dominant organisms in aerobic sewage treatment plants. If we assume that most organisms are present in suspended form and that most treatment plants have a hydraulic retention time around 6–12 h, degrading organisms must be able to adapt to increasing concentrations of heavy metals within a few hours if the efficiency of the plant is to be maintained. Furthermore, the cultivation of bacteria in a treatment plant involves a much more complicated process than our laboratory experiments conducted on defined media. Factors such as suspended solids concentration, sludge age, other metals present and the number of species present in the plant greatly influence the efficiency of waste-water treatment.

In conclusion it can be noted that the organism *Z. ramigera*, partly known for its ability to adsorb heavy metals, is sensitive to moderate concentrations of cadmium, cobalt and zinc thus indicating that the uptake process is of passive nature.

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Accumulation of heavy metal ions by *Zoogloea ramigera*.

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ACCUMULATION OF HEAVY METAL IONS BY *ZOOGLOEA RAMIGERA*

ABSTRACT

Biomass has been produced from glucose using the organism *Zoogloea ramigera* 115. This biomass has been used to remove copper, cadmium and uranyl ions from water solutions. The metal uptake was studied with two different methods; either by spectrophotometric measurements on the solutions after flocculation or by potentiometric measurements with amalgam electrodes in order to follow the entire complex formation. The metal-biopolymer interaction in 0.1 M NaClO₄ is practically the same as when no neutral salt was added. The metal uptake is dependent on pH and is selective. A reversible process suitable for metal complexation is described.

INTRODUCTION

Aqueous effluents emanating, for example, from the mining industry and metal plating factories contain dissolved heavy metals. If these discharges are emitted without treatment, they may have an adverse impact on the environment. Conventional methods for removing dissolved heavy metals include chemical precipitation, chemical oxidation and reduction, ion exchange, filtration, electrochemical treatment and evaporative recovery.

Using microorganisms as biosorbents for heavy metals offers a potential alternative to existing methods for detoxification and for recovery of toxic or valuable metals from industrial discharge water. In contrast to the intracellular uptake of metal cations by microorganisms, which involves an energy dependent system sensitive to pH and temperature, metal adsorption to negatively charged groups on the bacterial surface is rapid, reversible, and occurs whether or not a carbon or energy source is present in the medium¹. Algal cells have been successfully used as adsorbents for molybdenum² and uranium³. Two species of actinomycetes were found to have a considerable capacity to accumulate uranium in a process not affected by temperature and metabolic inhibitors⁴. Certain bacterial cells exhibit the property of metal accumulation by virtue of the inherent chemical nature of the cell wall or extracellular materials⁵.

Several factors interact in the removal of heavy metals from aqueous solutions using microorganisms. Among these factors are the specific surface properties of the organisms, the cell metabolism and the physico-chemical influence of the environment. A suitable substance for the attachment of cations is provided by the organisms excreting negatively charged polysaccharides which form a matrix around the cells. *Zoogloea*

ramigera, common in sewage plants, produces substantial amounts of extra-cellular polysaccharide when cultivated on a medium containing carbon source in excess⁶. The organism is sensitive to low concentrations of heavy metals⁷. However, despite this sensitivity to metal cations, it has successfully been used for metal adsorption^{8,9}. The genus *Zoogloea* and aspects of its physiology, etc. have been documented¹⁰.

In the present investigation, we describe a process for metal accumulation using *Zoogloea ramigera* biomass as an adsorbing agent.

MATERIALS AND METHODS

Production of biomass

The organism used in this study was *Zoogloea ramigera* 115 (ATCC 25935). The organism was maintained on Trypticase Soy Agar (BBL 11043). The cultivation medium had the following composition (g/l): glucose, 25; K_2HPO_4 , 2; KH_2PO_4 , 1; NH_4Cl , 1; $MgSO_4 \cdot 7H_2O$, 0.2; yeast-extract (Difco), 0.01. Glucose and $MgSO_4 \cdot 7H_2O$ were autoclaved separately.

The organism was cultivated in a 3-liter fermentor at 26°C. Agitation was 800 rpm and aeration was maintained at 0.5 VVM. Maximum biomass concentration (17.5 g/l) was obtained after 120 h of culture. Samples for metal uptake were withdrawn at different culture ages. The samples were centrifuged at 11,400 x g for 10 min and resuspended to initial volume with distilled water. The dry weight of biomass, including cells and extracellular polysaccharide was determined according to Norberg and Enfors⁶. (Briefly, after ultrasonication and centrifugation, the supernatant, containing polymer, was decanted and precipitated in alcohol. The precipitate was filtered off and the dry weight was determined gravimetrically. The remaining pellet, containing cells, was dried to constant weight. The sum of the dry weights corresponds to the dry weight of biomass.)

The uptake of the metal by the biomass was studied by means of two different techniques:

Metal uptake 1

The interaction between biomass and copper and cadmium ions, respectively, at different pH values was studied using a potentiometric technique¹¹. The emf, E, of galvanic cells of the following composition was measured:



(M = Cu and Cd, respectively.)

The solution in the left hand half cell was prepared in the following way:

To a volume V_0 of a solution S ($C_M \text{ M(ClO}_4)_2$), a volume, v , of a suspension of biomass containing 3.15 g biomass dry wt/l was added. By adding a volume, v_1 , of a solution $T_1(\text{C}_H \text{ HClO}_4)$, pH was adjusted to such a low value that the interaction between metal ions and biomass could be neglected (i.e., pH = 4 for Cu and pH = 5 for Cd). Subsequently, the pH of the solution was raised by titrating with a volume, v_2 , of a solution $T_2(\text{C}_A \text{ NaOH})$. After every addition of a titrant, pH and E were measured. In order to study the release of metal ion from the biopolymer, measurements were also made at further additions of HClO_4 . Titration series were performed with $v_0 = 20.0 \text{ ml}$; $v = 5.5 \text{ ml}$; $C_M = 3.22 \text{ mM}$ (Cu) and 3.25 mM (Cd). To investigate the influence of ionic medium on the metal-biomass interaction, measurements were performed in two series: without ionic medium and at the ionic strength 0.1 M using NaClO_4 as neutral salt.

To check if the presence of cadmium ions affects the biomass uptake of copper ions, potentiometric titration series were performed in which the solution S (see above) contained $3.22 \text{ mM Cu(ClO}_4)_2$ as well as $\text{Cd(ClO}_4)_2$. At pH = 5.50 (optimal for copper adsorption), the solution was filtered off from the precipitate and electrolyzed between platinum electrodes at 2.0 V for 2 hours, thus removing the remaining copper from the solution. The cadmium concentration was then determined by EDTA-titration at pH = 10 using ERIO-T as an indicator.

All chemicals were of analytical grade. Two phase copper amalgam (2% by weight) was prepared according to Fronaeus¹² by electrolyzing a copper sulfate solution between a platinum anode and a mercury cathode. Two phase cadmium amalgam (8% by weight) was prepared by dissolving cadmium in mercury. The amalgams were stored under dilute sulphuric acid. Stirring of the solution was provided by leading a stream of nitrogen gas into the titration vessel (Ingold 605 slightly modified to keep a little pool of amalgam at the bottom). In this way, oxidation of the amalgam was avoided. pH was measured with a Radiometer pHM52 equipped with Radiometer glass electrode

GK2402C. The temperature was $25.0 \pm 0.1^\circ\text{C}$. Equilibrium was attained within a few minutes after addition of titrant.

For the galvanic cell described above, the relation $E = E^0 - 29.58 \lg [M^{2+}]$ (*) was proven experimentally to be valid to within ± 0.2 mV for copper ion concentrations between 0.5 and 5 mM (for cadmium ± 0.1 mV between 0.5 and 5 mM concentrations). In the series, E^0 was determined from measurements at low values of pH at which the concentration of free metal, $[M^{2+}]$, approximately equals the total metal concentration, C_M .

From the measured values of E , the free metal concentration could then be calculated from eq. (*) and thereby the total amount of metal bound to biomass (or complexed otherwise, predominantly as hydroxo complexes in the actual pH-region) could be calculated. Thus the total fraction of metal bound, $(C_M - [M^{2+}])/C_M$ is obtained as a function of pH. To correct the measured results with respect to formation of metal-hydroxo complexes, identical titration series were performed without biopolymer. Thus the fraction of metal existing as hydroxo complexes was obtained as a function of pH as well. The fraction of metal bound to the biomass was then obtained as the difference between the two values.

Metal uptake 2

Metal salt solutions were prepared from $\text{UO}_2(\text{NO}_3)_2$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and $\text{CdSO}_4 \cdot 8/3 \text{H}_2\text{O}$, respectively. The washed suspension of biomass was added to the metal solution at room temperature. The pH in the mixture was adjusted to the desired value with 0.05 M sodium borate. The mixture was equilibrated for 15 minutes with continuous stirring. (By using sodium borate instead of sodium hydroxide, a smoother pH-adjustment was obtained.) The process was terminated by centrifugation at $11,400 \times g$ for 10 min, which thoroughly separated the flocculated biomass from the metal solution. The amount of metal adsorbed by the biomass was determined by measuring the residual metal concentration in the supernatant. In the case of copper and cadmium, the analyses were performed by an atomic absorption spectrometer (Varian AA - 5). Uranium was analyzed spectrophotometrically according to

Repeated uptake experiments on solutions containing copper and uranium.

To the solution which contained copper, biomass (final concentration $0.83 \text{ g dry wt.} \cdot \text{l}^{-1}$) was added during continuous stirring. Flocculation was initiated by adjusting pH to 5.5. Exposure continued for 15 min. The whole volume was centrifuged and the supernatant was decanted. A sample was withdrawn for analysis of remaining copper concentration. The residual copper solution was transferred back to the metal uptake reactor and fresh biomass was added. Again, flocculation occurred when pH was adjusted to 5.5. The same uptake procedure was repeated twice more. Remaining copper concentration was analyzed with AAS.

The uranyl solution was flocculated in the reactor by adding biomass (final concentration $0.83 \text{ g dry wt.} \cdot \text{l}^{-1}$). Flocculation occurred spontaneously at a pH of 3.1 at the first exposure to the biomass. After 15 min., the mixture was centrifuged. The second flocculation was initiated by adjusting pH to 3.5, the third to 4.0, the fourth to 4.5 and the fifth to 5.0. Remaining uranium concentration in the samples was analyzed spectrophotometrically.

Release of metal from *Z. ramigera*

Removal of cadmium and copper ions from biomass was studied in separate experiments. A copper solution ($0.475 \text{ g} \cdot \text{l}^{-1}$) was flocculated by adding biomass (final concentration $0.66 \text{ g} \cdot \text{l}^{-1}$) and then adjusting pH to 5.5. Exposure continued for 15 min. A withdrawn sample was centrifuged and the supernatant was analysed with respect to remaining copper. The mixture in the reactor was then adjusted to pH 5.0 by adding HCl. After exposure, a withdrawn sample was treated as described above. The mixture was adjusted in the same manner to pH 4.0, 3.0, and 2.0.

A solution containing cadmium ($0.490 \text{ g} \cdot \text{l}^{-1}$) was flocculated by adding biomass ($0.66 \text{ g} \cdot \text{l}^{-1}$) and adjusting pH to 6.5. The amount of bound cadmium was determined at pH 6, 5, 4 and 3 as described above for copper.

Solubility of polysaccharide

A copper solution was flocculated by adding biomass (final concentration 0.875 g dry wt./l) and then adjusting pH to 5.5. A withdrawn sample was centrifuged and the resulting supernatant was analyzed with respect to remaining polysaccharide according to the phenol-sulphuric acid method¹⁴.

To study the solubility of polysaccharide during the release period, 100 ml. of the flocculated copper solution was adjusted to pH 4.5 by adding HCl. Another portion was adjusted to pH 3.5. From each portion, samples were taken at 5, 20 and 60 min., respectively, after addition of HCl. These samples were centrifuged and the supernatant was analyzed with respect to remaining soluble polysaccharide. Mixing, using a magnetic stirrer, must be carefully controlled during the release phase. The stirring rate must not exceed 100-150 rpm if the flocs should remain in their aggregated state. At higher shear rates, the flocs are broken up mechanically and the polysaccharide dissolves more easily.

RESULTS

The effect of culture age on the uptake of Cd and Cu was studied initially. The results are shown in Fig. 1. The amount of metal removed from the solution reached a maximum when cultures 6-8 days old were used. The following uptake experiments were performed with biomass harvested within this period of time. Biomass could be stored in a deep-freeze until the time of usage. Thawed biomass had the same uptake capacity as freshly produced biomass.

The biomass uptake of copper and cadmium as a function of pH was calculated from the potentiometric measurements. The results are represented in Figs. 2 and 3 at different ionic strengths. As can be seen from the data plotted, copper is more effectively adsorbed to the biomass than cadmium at low values of pH. It is also obvious that a moderate addition of neutral salt has a practically negligible influence on the interaction between biomass and metal ions. From the measurements without biomass, the extent of hydroxo complex formation was determined. The following approximate evaluation of the distribution of metal on free metal ion, metal-hydroxo complexes and metal bound to biomass in Table 1 is based on Figs. 2 and 3. The measurements with both copper and cadmium in the solution showed that:

- 1) The presence of cadmium ions did not affect the biomass uptake of copper ions.
- 2) Within the experimental limits of accuracy ($\pm 2\%$), no cadmium was removed from the solution at pH = 5.5.

The flocculation step in the process was studied with respect to metal uptake rate. As we used centrifugation to separate flocculated biomass from the metal solution, this markedly extends the time of exposure. However, equilibrium was reached within 15 min. and prolonged exposure time did not increase removal of copper or cadmium (Fig. 4).

The effect of metal concentrations on the uptake of metal was studied by adding equal amounts of biomass to solutions with different copper concentrations (Fig. 5).

Fig. 6 shows the result from repeated flocculation of a solution containing copper. The copper concentration decreased from 0.525 g/l to below 0.01 g/l after three successive flocculation steps. Further exposure to the biomass only marginally improved the removal of copper from the solution. Results from successive flocculations of a uranium solution are presented in Fig. 7. As can be seen, uranium is effectively adsorbed by the biomass already at pH 3.1.

Formation of metal-hydroxo complexes

The following calculations are based on stability constants taken from Smith and Martell:¹⁵ Critical Stability Constants, referring to zero ionic strength and 25°C, and agree well with those reported by Baes and Mesmer.¹⁶ The dominating cadmium-hydroxo complex at the pH values relevant in the present investigation is reported to be CdOH^+ . From $\text{Cd}^{2+} + \text{OH}^- \rightleftharpoons \text{CdOH}^+$ with $\log \beta_1 = 3.9$, we get $[\text{CdOH}^+]/[\text{Cd}^{2+}] = 10^{-10.1}/[\text{H}^+]$. Thus, at pH = 6, about 0.01% of cadmium should exist as CdOH^+ , at pH = 7, about 0.1%, and at pH = 8, about 1%. These values are in good agreement with the results from our measurements without biomass. If $\log K_S = -14.35$ is assumed correct for the solubility product of cadmium hydroxide and $[\text{Cd}^{2+}] = 2\text{mM}$, a precipitation should occur at pH ≈ 8.2 . In our measurements without polysaccharide, no precipitation was obtained until pH ≈ 9 , probably due to super saturation.

The dominating copper (II) hydroxo complexes at moderate pH values are reported to be CuOH^+ and a dinuclear complex $\text{Cu}_2(\text{OH})_2^{2+}$. From $\text{Cu}^{2+} + \text{OH}^- \rightleftharpoons \text{CuOH}^+$ with $\lg \beta_1 = 6.3$, we obtain $[\text{CuOH}^+]/[\text{Cu}^{2+}] \approx 0.2\%$ at pH = 5 and 2% at pH = 6. From $2\text{Cu}^{2+} + 2\text{OH}^- \rightleftharpoons \text{Cu}_2(\text{OH})_2^{2+}$ with $\lg \beta_{22} = 17.7$ and given that $[\text{Cu}^{2+}] = 1.8\text{ mM}$, we get $[\text{Cu}_2(\text{OH})_2^{2+}]/[\text{Cu}^{2+}] \approx 0.1\%$ at pH = 5 and about 9% at pH = 6.

Thus, from the literature data, the total fraction of copper bound as hydroxo complexes can be estimated to be about 0.4% at pH = 5 and about 20% at pH = 6. Our measurements give a value of $(15 \pm 5)\%$ hydroxo complexes at

pH = 6, in good agreement with the literature data. If the value $\log K_S = -19.3$ is assumed correct for the solubility product of copper (II) hydroxide and $[\text{Cu}^{2+}] = 2 \text{ mM}$, precipitation of copper hydroxide should occur at $\text{pH} \approx 5.7$. In our measurements without polysaccharide, precipitation was not obtained until the pH was considerably higher, possibly again due to super saturation.

Separation methods

For the separation of flocculated biomass from the solution, centrifugation was the separation method mainly used, but settling under the natural force of gravity could also be employed. In one experiment with copper, a comparison was made between these methods. A metal solution containing 0.495 g/l of Cu^{2+} was flocculated by adding 0.7 g of biomass (dry wt)/l solution and adjusting pH to 5.5. In one case, at the end of the exposure time, a sample was pipetted out of the continuously stirred solution and centrifuged at $11,400 \times g$ for 10 min before the supernatant was decanted and used for analysis of remaining copper. In the second case, the flocs in the reactor were allowed to settle for 10 min after the exposure time. Then a second sample was withdrawn from the clear solution by a pipett.

Both samples were acidified by adding 0.05 ml concentrated nitric acid, which released traces of copper adsorbed to unsettled small flocs. AAS analysis showed that both samples contained 0.32 g Cu^{2+} /l. Thus, both methods can be used for the purpose of separation, but centrifugation is advantageous when the pellet volume is considered. Centrifugation produces a pellet which is firm and rigid, while sedimentation by force of gravity produces a slurry.

Release of metals from biomass

Acid treatment was used to release metals from the flocculated particles which had been formed during exposure to metals. In Fig. 8, data are plotted for separate experiments with cadmium and copper. Cadmium is more easily released from the aggregates than copper. Both metals can

effectively be separated from the biomass by increasing the acidity. Furthermore, acid treatment of the flocs containing metals only marginally dissolved the polysaccharide fraction of the biomass if the stirring rate was sufficiently low (<150 rpm). The length of the release period only slightly affected the solubility of the polysaccharide; after 5 min., 3.9%, after 20 min., 4.4% and after 60 min., only 4.7% of the polysaccharide was dissolved. No difference, regarding solubility of polymer, was found if release was performed at pH 4.5 or 3.5. The remaining biomass, free of metal, was thoroughly stirred with a portion of H_2O . This treatment dissolved the polymer and its rheological characteristics were restored. The biomass was either transferred back to the storage vessel or directly used for renewed flocculation.

Description of the process

Recycling of metal-adsorbing biomass can be achieved in the following way. Biomass, consisting of bacterial cells and polysaccharides, is added to a heavy metal solution at a pH value which supports floc formation. Appropriate pH control can be achieved by automatic titration using a pH-meter connected to a titrator. The resulting flocs are large (2-8 mm) and separation from the aqueous metal solution is therefore easily performed by centrifugation. The supernatant is decanted and discharged if considered free of heavy metals. However, if the concentration of heavy metal is still too high, flocculation can be repeated until the concentration of toxic metal in the solution is reduced to an acceptable level. The pellet, consisting of bacterial biomass and adsorbed metal ions, is diluted with an equal volume of distilled water and broken up mechanically into a slurry. This slurry is acidified with HCl and equilibrated for 15 min. During this treatment, the flocs release the previously bound metal. Separation of bacterial flocs from dissolved metal cations is performed through centrifugation. The remaining pellet, consisting of

regenerated biomass, is transferred to and resuspended in the reservoir containing fresh biomass. The biomass is stirred which ensures a homogeneous culture. Any decrease in pH, due to the addition of acidic biomass is compensated for by the addition of NaOH. Floc rigidity is strongly dependent on the acidity of the solution and stirring rate; therefore, these parameters have to be carefully controlled when acid is added to the slurry of flocs.

Discussion

An optimal process for the recovery of metals from water solutions using an adsorbing agent should fulfil the following demands:

- 1) The phases of uptake and release of metal should be efficient and rapid.
- 2) The adsorbing agent should be produced at a moderate cost and, if possible, reused.
- 3) A cheap, efficient and rapid method for separation of metal-adsorbent aggregates should be possible.
- 4) Of course, it is also economically favourable if the reagent used for releasing the metal is cheap and the losses of adsorbent at the release phase are low.
- 5) If the water solution contains different metals and a separation of these metals is required, it is a great advantage if the adsorption process can also be used for this purpose.

A critical examination of the process outlined in the present report with respect to these demands shows:

- 1) The uptake and release phases are rapid--within less than 15 min., the processes are completed. Desired efficiency in the uptake process can be achieved by repeated flocculation. High efficiency is brought about in the release phase by lowering pH.
- 2) It is possible to produce biomass at a moderate cost using *Zoogloea*

ramigera. A process for reuse of biomass is described above.

3) The separation step is rapidly and profitably performed by flocculation followed by centrifugation. Alternatively, sedimentation by gravity alone can be used. The flocs produced are rigid and porous, thereby simplifying the elimination of water.

4) The release of metal from biomass is achieved by an inexpensive and simple reagent, hydrochloric acid. The losses of adsorbent in the release phase can be kept low.

5) In the present investigation it has been shown that a separation of copper and cadmium ions can be achieved by using the selective metal adsorption to biomass at different pH-values.

Thus the process reported is suitable for removing, for example, copper and cadmium from water. The capacity for biomass to complex metal ions is extremely high.

A study regarding the structure of the polysaccharide produced from glucose by *Z. ramigera* 115 has recently been published.¹⁷ The polysaccharide is reported to be composed of galactose, glucose and pyruvate units.

Acknowledgments

This work was sponsored by the Biotechnology Research Foundation and the Swedish Board for Technical Development. We also thank Mrs. Ingegerd Lind for skilful assistance in drawing the figures.

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Table 1. Distribution of copper and cadmium; free metal ion, hydroxo complexes and metal bound to biomass.

pH	% of Cu as			% of Cd as		
	Cu ²⁺	hydroxo complexes	bound to biomass	Cd ²⁺	hydroxo complexes	bound to biomass
4.0	100	0	0	100	0	0
4.5	97	0	3	100	0	0
5.0	79	1	20	100	0	0
5.5	42	6	52	99	0	1
6.0	29	15	56	91	0	9
6.5				68	0	32
7.0				63	0	37
7.5				61	0	39
8.0				58	1	41

Legends to figures

Fig. 1. Effect of culture age on the uptake of cadmium and copper. The concentration of biomass was 0.60 - 0.67 g dry wt/l (for Cu) and 0.63 - 0.75 g dry wt/l (for Cd). The metal concentration was 0.48 g/l (Cu) and 0.49 - 0.81 (Cd). ● copper, ○ cadmium

Fig. 2. Bound copper ($(C_{Cu} - [Cu^{2+}])/C_{Cu}$) as a function of pH.

□ Biomass added - no neutral salt

■ Biomass added - ionic medium (0.10 M $NaClO_4$)

○ No biomass added - no neutral salt

● No biomass added - ionic medium (0.10 M $NaClO_4$)

$C_{Cu} = 2.4 - 1.7$ mM

Biomass: 0.68 - 0.50 g dwt/l solution

Solution volume: 25 - 35 ml.

Fig. 3. Bound cadmium ($(C_{Cd} - [Cd^{2+}])/C_{Cd}$) as a function of pH.

□ Biomass added - no neutral salt

■ Biomass added - ionic medium (0.10 M $NaClO_4$)

○ No biomass added - no neutral salt

● No biomass added - ionic medium (0.10 M $NaClO_4$)

$C_{Cd} = 2.4 - 1.8$ mM

Biomass: 0.68 - 0.50 g dwt/l solution

Solution volume: 25 - 35 ml.

Fig. 4. Time course of the uptake of cadmium and copper by biomass. The concentration of biomass was 0.42 - 0.66 g dry wt./l (for Cu) and 0.66 g dry wt./l (for Cd). The metal concentration was 0.48 g/l (Cu) and 0.7 g/l (Cd). ● copper, ○ cadmium

Fig. 5. Effect of copper concentration on the uptake of copper by biomass. The biomass concentration was 0.83 g dry wt./l.

Fig. 6. Effect of repeated flocculations on a copper solution. The biomass concentration was 0.83 g dry wt./l. Equilibration pH was 5.5.

Fig. 7. Effect of repeated flocculations on a uranium solution. The concentration of biomass was 0.83 g dry wt./l.

Fig. 8. Release of previously adsorbed metal by increasing acidity.

○ cadmium

● copper

FIG. 1

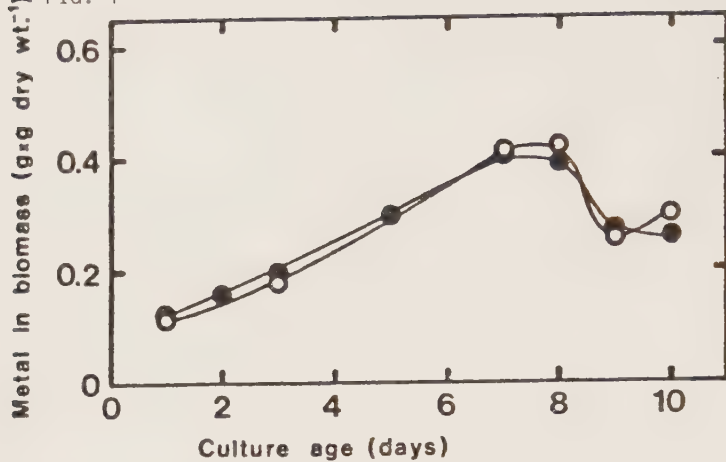


FIG. 2

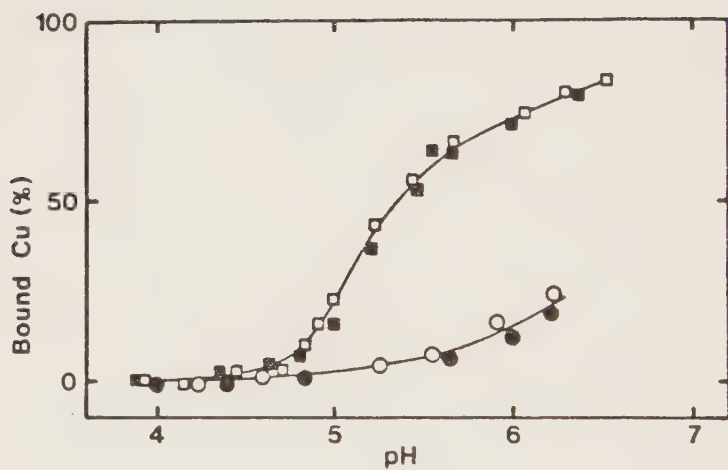


FIG. 3

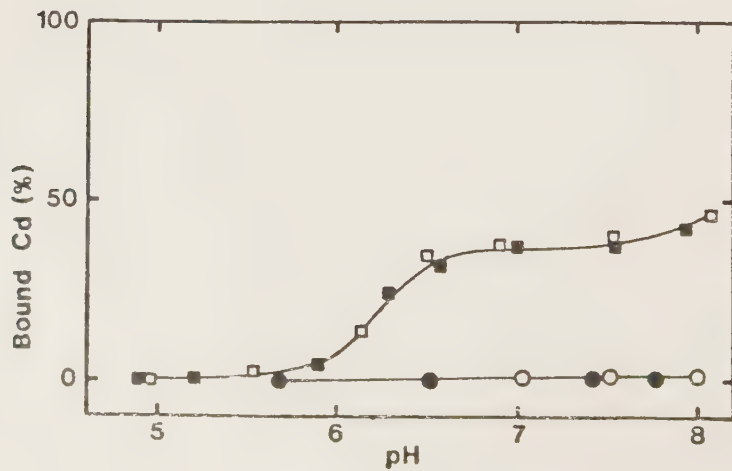


FIG. 4

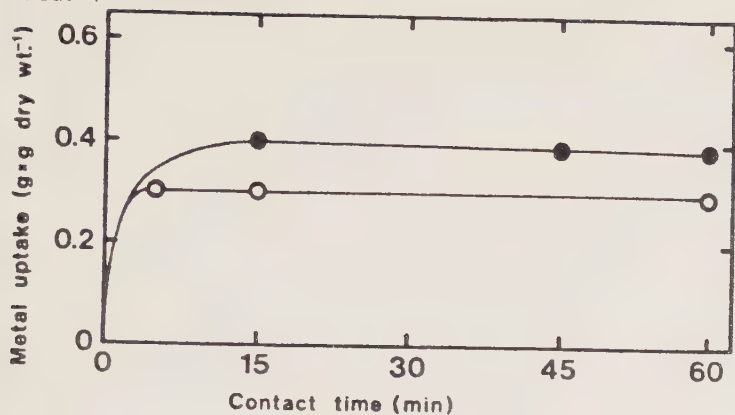


FIG. 5

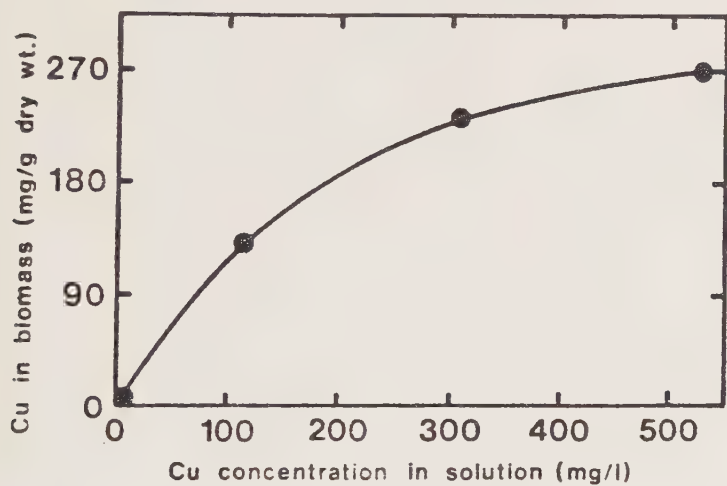


FIG. 6

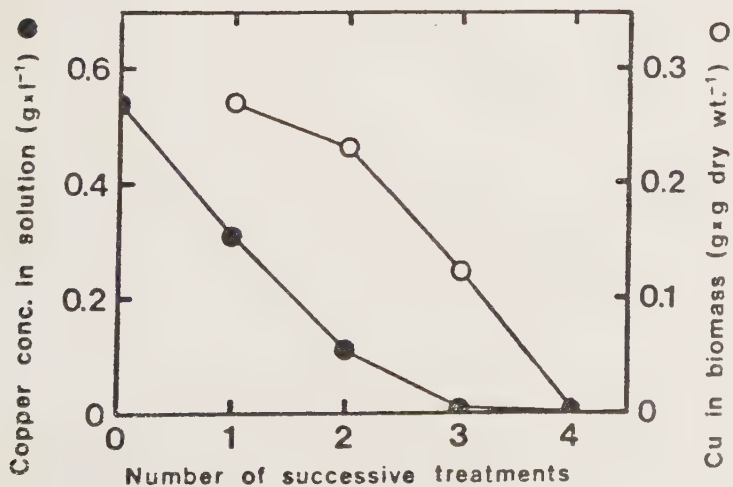


FIG. 7

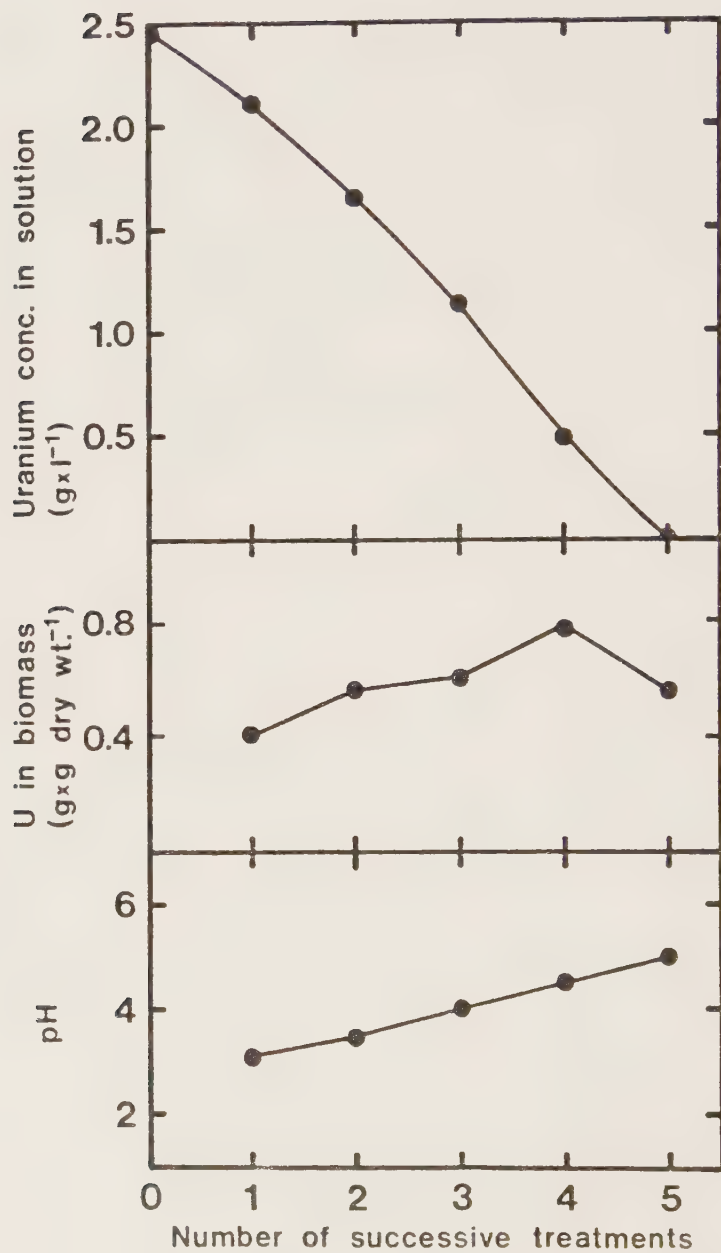
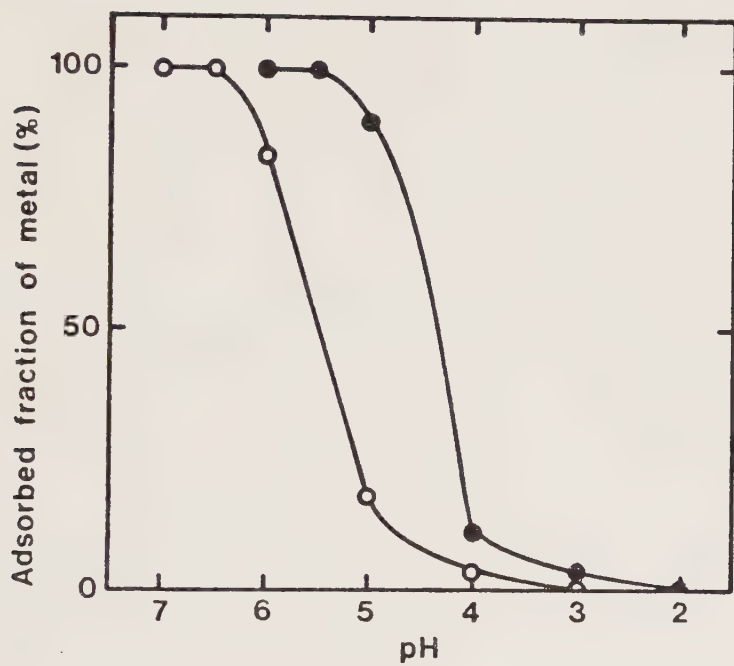


FIG. 8



Selective accumulation of heavy metal ions by *Zoogloea ramigera*.

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SELECTIVE ACCUMULATION OF HEAVY METAL IONS BY *ZOOGLAEA RAMIGERA*.

ABSTRACT

Biomass, consisting of bacterial cells and an acidic polysaccharide, produced by the bacterium *Zoogloea ramigera* 115 has been used as adsorbing agent for heavy metal cations. Adsorption of metal cations to the biomass results in the formation of aggregated particles or flocs which can easily be separated from the metal solution. Treatment of solutions containing different heavy metal ions indicates that selective uptake of metals can be performed by adjusting pH to appropriate levels. Aluminium was found to be more effective than magnesium and potassium in preventing adsorption of copper to the bacterial biomass.

INTRODUCTION

The accumulation of heavy metal ions from industrial effluents by using microbial biomass as adsorbing agents has, during the last few years, shown promising results. Further developments regarding process design and the use of more efficient organisms derived through genetic manipulation could make bioadsorption of heavy metals an alternative to conventional methods for metal removal from process effluents.

Both algae and bacteria can selectively accululate metal ions from aqueous systems. The green algae, *Chlorella regularis*, has been shown to prefer uptake of uranyl ions when exposed to a mixture of heavy metal ions.¹ Accumulation of uranium, by using *Saccharomyces cerevisiae* and *Pseudomonas aeruginosa* as bioadsorbing agents, has also shown fruitful results.² Two species of actinomycetes have been reported to exhibit high accumulation abilities when exposed to uranium.³

We have previously reported that biomass produced by *Zoogloea ramigera* can be used to recover or remove heavy metal ions from aqueous process effluents.⁴ The organism produces an extracellular polysaccharide when cultivated in a medium containing carbon source in excess.⁵ This polysaccharide characteristically promotes flocculation when exposed to a heavy metal solution. The metal-containing flocs or particles formed upon aggregation of biomass are easy to separate from the aqueous solution.

In this paper, we report on the selective accumulation of heavy metals by the biomass produced by *Z. ramigera*. We have also investigated the influence of cations on flocs which previously had accumulated copper.

MATERIALS AND METHODS

Organism

The organism used was *Zoogloea ramigera* strain 115 (ATCC 25935). The bacterium was maintained on Trypticase Soy Agar (BBL 11043) slants.

Biomass production

The biomass used for metal accumulation experiments was prepared by cultivating the organism on the following medium ($\text{g}\cdot\text{l}^{-1}$): glucose, 25; K_2HPO_4 , 2; KH_2PO_4 , 1; NH_4Cl , 1; $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 0.2; yeast extract (Difco), 0.01. Glucose and $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ were autoclaved separately.

The organism was cultivated in a 3-litre fermenter (Chemoferm, Sweden) at 26°C . Agitation was 800 rpm, aeration was maintained at 0.5 VVM. Maximum biomass concentration ($17.5 \text{ g}\cdot\text{l}^{-1}$), on dry weight basis, was obtained after 120 h of culture. The main part ($15 \text{ g}\cdot\text{l}^{-1}$) of the harvested biomass consisted of polysaccharide and the remaining part ($2.5 \text{ g}\cdot\text{l}^{-1}$) was bacterial cells. The biomass was stored in a freezer until needed.

Metal uptake experiments

Biomass, produced by *Z. ramigera*, was added to 200 ml of metal ion solution. The uptake experiments were performed at room temperature in a reactor equipped with a pH-electrode connected to a pH-meter and a titrator, to allow adjustment of pH. The reactor was placed on a magnetic stirrer; the stirring rate was 700 rpm if not otherwise stated. The metal salts used were $\text{CuCl}_2 \times 2\text{H}_2\text{O}$, $\text{CdSO}_4 \times 8/3 \text{ H}_2\text{O}$ and $\text{UO}_2(\text{NO}_3)_2 \times 6\text{H}_2\text{O}$. All chemicals used were of analytical grade.

I. Successive flocculation of a solution containing cadmium, copper and uranium.

The mixture of dissolved metals and biomass was flocculated in the metal uptake reactor by adding 0.175 g (dry wt.) of biomass. The initial cadmium concentration was $0.500 \text{ g}\cdot\text{l}^{-1}$, the copper concentration was $0.308 \text{ g}\cdot\text{l}^{-1}$ and the uranium concentration, $1.28 \text{ g}\cdot\text{l}^{-1}$. Equilibration occurred at pH 3.1. No adjustment of pH was necessary to achieve flocculation. After exposure (15 min.), the flocs were separated from the solution by centrifugation ($11,400 \times g$, 10 min.). A portion of the supernatant (20 ml) was withdrawn and analyzed with respect to cadmium, copper and uranium in solution. The remaining supernatant was used for repeated flocculation. Successive flocculations were then performed at pH = 3.5, 4.0, 4.75, 5.50, 6.0, and 6.5, respectively. The concentrations of cadmium and copper in the solutions were determined with AAS. Uranium was measured spectrophotometrically.⁶

II. Flocculation of a solution containing cadmium and copper followed by release of the metals and reuse of the adsorbing matrix.

The metal mixture was pipetted into the metal uptake reactor. Initial copper concentration was $0.245 \text{ g}\cdot\text{l}^{-1}$ and the cadmium level was $0.345 \text{ g}\cdot\text{l}^{-1}$. Mixing was achieved by continuous stirring. Biomass was added to a final concentration of $0.65 \text{ g dry wt.}\cdot\text{l}^{-1}$, and flocculation was obtained by adjusting pH to 5.75. Exposure, separation of flocs and analysis of remaining metal concentration was performed as described previously.

After equilibration, 40 ml of the slurry containing both metals was centrifuged ($11,400 \times g$, 10 min). The supernatant was discarded. Distilled water was added to the sediment to restore the initial volume. HCl (0.1 M) was added to the slurry. Gentle mixing was maintained by adjusting the stirring rate to 150 rpm. Equilibration pH was 3.2. The treatment continued for 15 min. After the equilibration, the sample was centrifuged ($11,400 \times g$, 10 min) and the supernatant was analyzed with respect to cadmium and copper. The residual pellet from the experiment described above was dissolved in the aqueous solution containing copper ($0.245 \text{ g}\cdot\text{l}^{-1}$) and cadmium ($0.345 \text{ g}\cdot\text{l}^{-1}$). This was done in order to determine whether this biomass could be used for reaccumulation of heavy metals without loss of capacity. Flocculation was initiated by adjusting pH to 5.75. Exposure, separation and analysis of metal concentration were performed as described previously. The experiments, described above, were also performed for each metal at a time at pH = 5.5 and 6.5 for copper and cadmium, respectively.

III. Influence of mono-, di-, and trivalent cations on uptake of copper.

When studying the effect of Na^+ , Mg^{2+} and Al^{3+} on Cu^{2+} uptake, a copper solution (8.4 mM) was supplemented with NaCl to a final concentration of 10 mM . To this mixture, 0.175 g (dry wt.) biomass was added and exposure continued for 15 min at pH 5.5 under continuous stirring. Separation of flocculated biomass was performed by centrifugation ($11,400 \times g$, 10 min). Copper in the supernatant was measured with AAS.

The same procedure was repeated for magnesium, which was added as $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (10.5 mM) and aluminium as $\text{Al}_2(\text{SO}_4)_3 \cdot 16\text{H}_2\text{O}$ (10 mM).

The copper solution, without addition of external cations, was also flocculated and the biomass uptake capacity in this sample was compared to the others.

IV. Influence of K^+ , Ca^{2+} , Mg^{2+} and Al^{3+} on biomass flocculated with copper.

A copper solution, containing $0.59 \text{ g Cu} \cdot \text{l}^{-1}$, was flocculated in the metal uptake reactor by adding 0.175 g (dry wt.) biomass and adjusting pH to 5.5. Exposure continued for 15 min, whereafter 20 ml of the slurry was withdrawn and centrifuged prior to analysis of remaining copper in solution. Concentrated KCl-solution was then added to the remaining sample in the reactor to give a concentration of 1 mM. After 15 min, another sample was withdrawn and centrifuged and its copper concentration was determined by using AAS. In the same manner, the KCl concentration was consecutively raised to 10 and 25 mM. Samples were withdrawn after each addition of KCl and analyzed with respect to copper in solution. The other cations, added as CaCl_2 , $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and $\text{Al}_2(\text{SO}_4)_3 \cdot 16\text{H}_2\text{O}$ were each supplemented to a freshly flocculated copper solution in the same way as described for KCl.

RESULTS

I. Successive flocculation of a solution containing cadmium, copper and uranium is shown in Fig. 1. Uranium was initially most effectively adsorbed to the biomass, and within the first four flocculations the uranium concentration in the solution declined from 1.28 to $0.0005 \text{ g U} \cdot \text{l}^{-1}$. The mean uptake of uranium during these four flocculations was $0.37 \text{ g U} \cdot \text{g biomass}^{-1}$. Copper was most effectively adsorbed during the fourth and fifth flocculation. Equilibration pH during these flocculations was 4.75 and 5.50, respectively, which is suitable for copper adsorption. Cadmium on the other hand, was most effectively removed at the sixth flocculation ($0.171 \text{ g Cd} \cdot \text{g biomass}^{-1}$) when pH was adjusted to 6.5. The total amount of metal removed was 2.06 g after adding a total of 6.13 g biomass.

II. Results from the flocculation of a solution containing cadmium and copper by adding the adsorbing agent, subsequent removal of the adsorbed metal ions from the flocs, and reexposure of the regenerated biomass to the aqueous mixture containing cadmium and copper are presented in Fig. 2.

The metal uptake values obtained upon reexposure of used and regenerated biomass to a metal solution revealed that the acid treatment did not affect the metal binding capacity of the biomass.

From the flocculation experiments with each metal separately, the metal uptake values were calculated to be 0.323 and 0.223 (g metal·g dry wt. biomass⁻¹) for copper and cadmium, respectively. Copper is thus more efficiently removed than cadmium.

III. The effects on the biomass uptake capacity of copper produced by other cations in the copper solution before biomass was added are shown in Table 1. Trivalent aluminium was more effective than divalent magnesium and monovalent sodium in preventing copper from being adsorbed to the biomass.

IV. Addition of potassium, calcium or magnesium to a slurry containing biomass, which had earlier adsorbed copper, did not affect the distribution between adsorbed copper and copper in solution (Fig. 3). When aluminium was added, however, the amount of soluble copper decreased (Fig. 3). This effect may depend on precipitation of $\text{Al}(\text{OH})_3$ and co-precipitation of copper.

DISCUSSION

The biomass produced by *Z. ramigera* 115 exhibits a potential ability to accumulate metal ions. Our results show that the biomass can be used to accumulate heavy metal ions selectively.

Friedman and Dugan⁷ suggested the application of the organism in treatment of industrial effluents. The results from this work and from earlier reports from our laboratory support this potential application.

Dugan and Pickrum⁸ found that *Z. ramigera* 115 adsorbed metal ions from mine water. The affinity of the biomass for ion adsorption was considered to be $\text{Fe} > \text{Cu} > \text{Co} > \text{Ni}$.

The metal uptake capacity of the biomass on a weight basis is very different for different metals. However, a comparison on molar basis yields a better correlation. Equal molar amounts of copper and uranium can be adsorbed to the biomass. Recent structure studies of the polymer produced by *Z. ramigera* 115 suggests that the polysaccharide is a weakly acidic polymer composed of galactose, glucose and pyruvate in a proposed molar ratio of 11:3:1.5.⁹ The presence of pyruvate forms a basis for binding. The carboxylate group is known to form bonds with different metal ions. Other potential bonding sites, like the oxygen atom on the sugar ring and certain alcoholic groups of polysaccharides have been shown to interact with metal ions as well.¹⁰ Furthermore, cation adsorption to negatively charged ligands on the organism's cell surface must be considered in our experiments, since bacterial cells were present in the biomass used for metal accumulation. Ligands in the cell surface responsible for cationic interactions are e.g. phosphoryl-, carboxyl-, sulphhydryl-, and hydroxyl groups.

The results obtained from the treatment of an aqueous solution containing cadmium, copper and uranium with biomass confirms the observation that adjustment of pH can be used as a tool for obtaining selective metal uptake. Uranium is effectively adsorbed to the biomass already at pH-values around 3.5, copper around pH 5.5, and cadmium at pH 6.5.

However, the basis for selective metal adsorption has to be developed further if treatment of natural effluents is to be achieved. Most industrial effluents contain a spectrum of ions. Most of these are non-valuable ions which may interact strongly with the adsorbing agent and thus more or less inhibit adsorption of valuable metals.

During the process phase, when the biomass with adsorbed metal is treated with acid, the metal is rapidly released. If the biomass is not stirred too vigorously, however, a relatively small fraction of biomass is lost during the subsequent separation. The comparatively slow kinetics of this solvation process can thus be used to minimize losses of biomass during the regeneration phase.

It can be concluded that in a process for the removal of heavy metals from aqueous solution based on adsorption of ions to microbial biomass, control of several parameters is necessary. However, when treatment of natural effluents is considered, the proposed process must be simple in order to achieve reliable performance during long-term operation.

The knowledge gained from our laboratory experiments is at the present time not easily transferred to the treatment of industrial effluents. However, current research in our laboratory is oriented towards developing a process applicable to rough field conditions.

Acknowledgements

This study was sponsored by the Biotechnology Research Foundation and the Swedish Board for Technical Developments. We are very grateful to Karl Mohlin for drawing the figures.

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Table 1. Adsorption of copper to biomass in the presence of other cations at pH = 5.5.

Cation	Conc. of cation (mM)	Copper conc. in solution after exposure ($\text{g}\cdot\text{l}^{-1}$)	Uptake of copper by biomass ($\text{g}\cdot\text{g}^{-1}$ dry wt.)
none	-	0.252	0.323
Na^+	10	0.256	0.319
Mg^{2+}	10.5	0.268	0.305
Al^{3+}	10	0.324	0.241

Legends to figures

- Fig. 1 Successive removal of metal ions from an aqueous solution containing cadmium, copper and uranium.
- Fig. 2 Complete metal treatment cycle, including exposure of biomass to an aqueous solution containing cadmium and copper, treatment with hydrochloric acid followed by reexposure of biomass to cadmium and copper.
- Fig. 3 Influence of K^+ , Ca^{2+} , Mg^{2+} , and Al^{3+} on copper previously adsorbed to the microbial biomass.

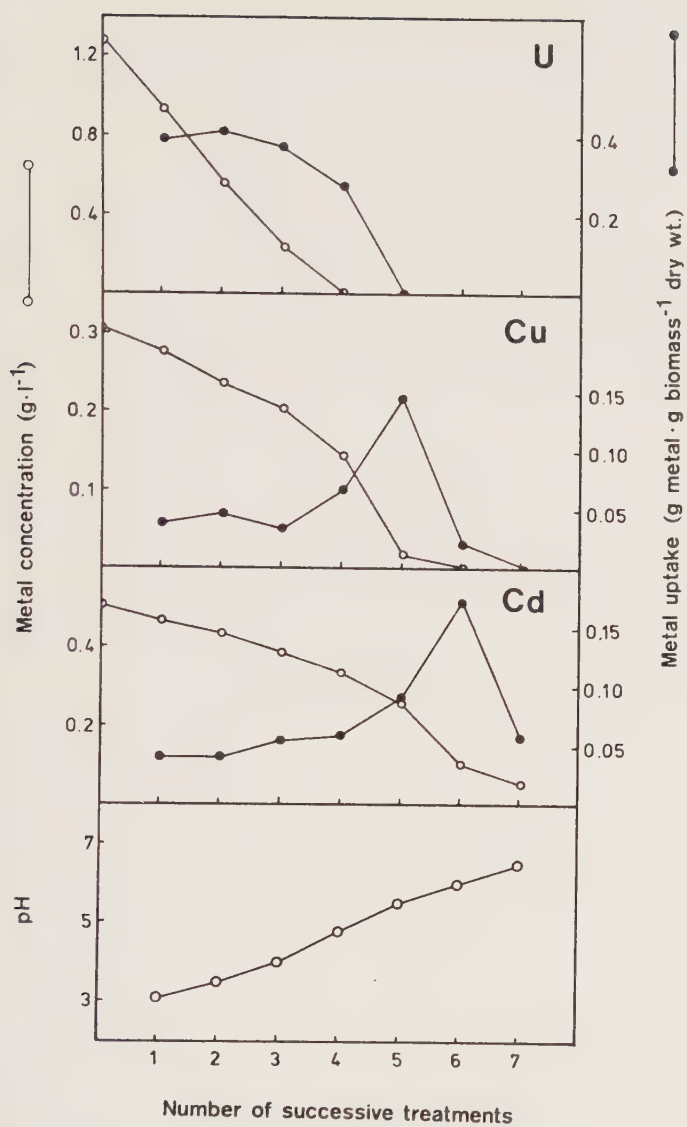


Fig.1

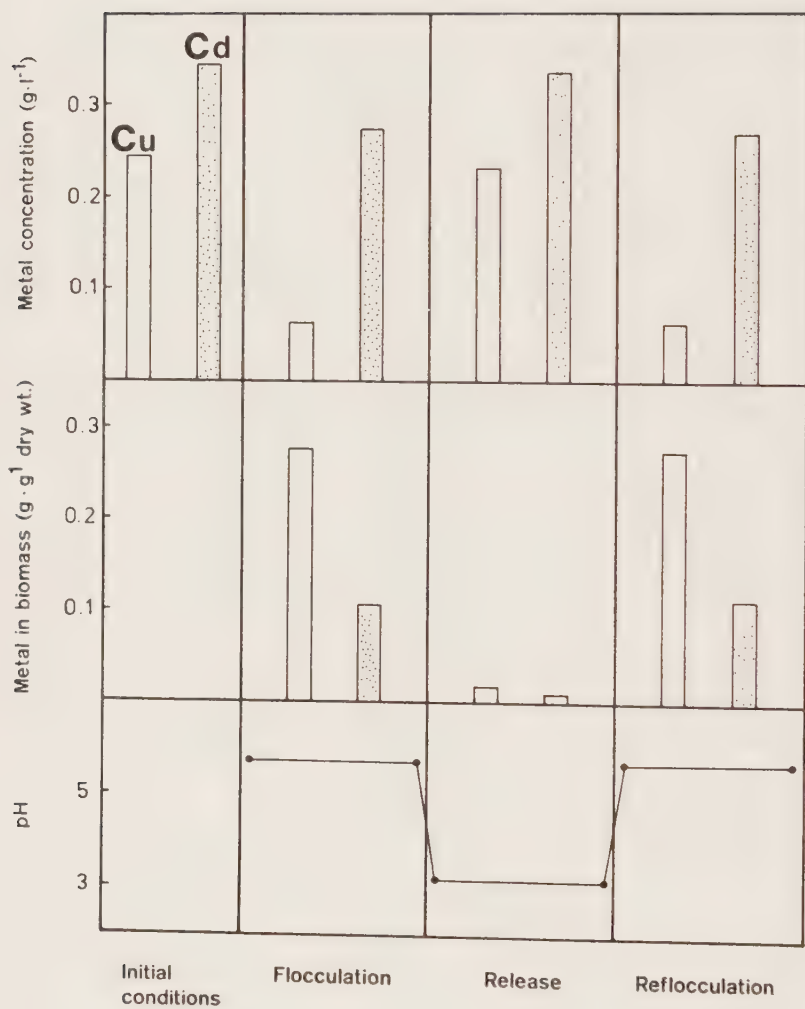


Fig.2

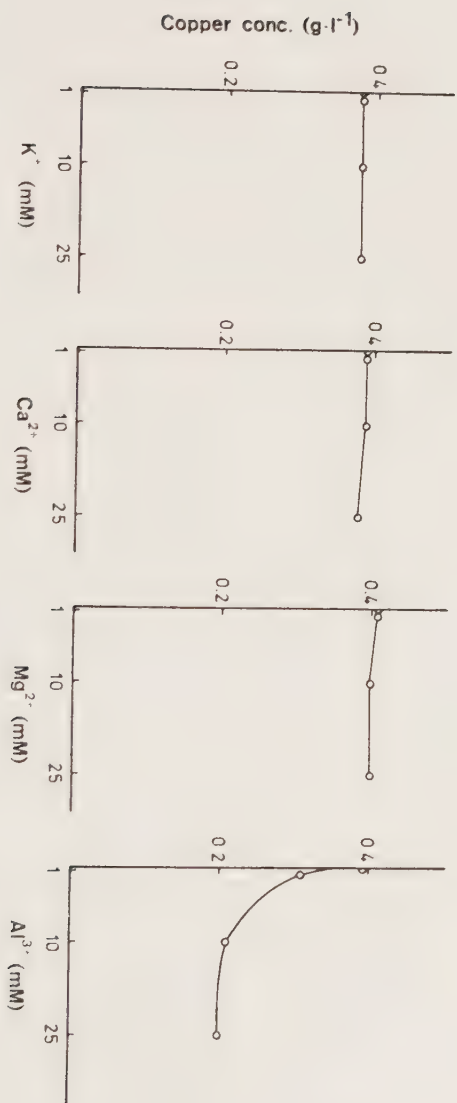


Fig. 3

Heavy metal adsorption by *Zoogloea ramigera* in comparison to other adsorbing agents.

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Heavy metal adsorption by *Zoogloea ramigera* in comparison to other adsorbing agents.

ABSTRACT

The adsorption of copper (II) ions by *Zoogloea ramigera* has been compared to the adsorption to three other agents using spectrophotometric and potentiometric methods. *Zoogloea ramigera* was found to be a considerably more effective adsorbing agent than polyacrylic acid, carboxymethylcellulose and another bacterial species, *Pseudomonas denitrificans* in the order mentioned. The adsorption of cadmium ions by *Z. ramigera* and polyacrylic acid were also compared. The selective adsorption of copper in a mixture of copper and cadmium exhibited by *Z. ramigera* is not shown by polyacrylic acid.

Introduction

Human activity has resulted in elevated concentrations of dissolved heavy metals in the environment. Increased amounts of, e.g. cadmium and copper have been released from the crust of the earth mainly due to mining activities, to precipitation from the burning of oil and coal and to the leaching effect caused by the elevated hydrogen ion concentration in rain-water.

During the last decade, increased interest has been shown for metal ion adsorption to viable or non-viable organisms. Although viable microorganisms are able to concentrate and accumulate a wide spectrum of metal species via metabolically mediated processes (Silverman and Ehrlich, 1964)¹, passive adsorption of metal ions by cells and cellular components seems to have considerable advantage for process application (Strandberg et al., 1981)². The sugars and derivatives of them in bacterial exopolysaccharides can, e.g., contain reactive carboxyl and hydroxyl groups. The negatively charged polysaccharides surrounding bacterial cells and alginic acids from brown algae exhibit interesting interactions with metal ions³. Corpe reports that extracts from strains of marine pseudomonads form precipitates when exposed to iron, copper and lead⁴. From experiments with *Z. ramigera* 115 and different metal ions, Ikeda et al. claim that Fe^{2+} and Fe^{3+} are the most efficient ions in precipitating the purified polysaccharide⁵. Bacteria surrounded by extracellular polysaccharides have been reported to exist as dispersed particles in ocean water⁶. McKnight et al. conclude that such particles can be formed by precipitation of polymers excreted by algae and bacteria⁷.

A potential application of charged polysaccharides is their interaction with pollutants. Metal ions may be removed from solution by adsorption to sites on bacterial extracellular polymers⁸.

Polyacrylic acid has for quite a long time been known to form complexes with metal ions. Thus Wall and Gill concluded from spectrophotometric and polarographic measurements that Cu^{2+} forms a chelate involving two carboxylate groups⁹. This conclusion is supported by McLaren et al. by results from potentiometric studies¹⁰.

Recent work in our laboratory has shown that the bacterium *Zoogloea ramigera*, common in sewage treatment plants, can adsorb substantial amounts of dissolved metal ions¹¹. The bacterium produces an extracellular

polysaccharide when cultivated in appropriate media¹². According to Ikeda et al., the polysaccharide produced is composed of glucose, galactose and pyruvate⁵. The bacterial cells and the polysaccharide are used together as an adsorbing agent in a simple step-wise process for metal accumulation. The metal ions adsorbed to the biomass can then be released by adding acid. Thus, the regenerated biomass can be reused for further metal ion adsorption.

In the present work, the metal uptake capacity of biomass produced by *Z. ramigera* is compared to carboxymethylcellulose, polyacrylic acid and another bacterial species, *Pseudomonas denitrificans*. The metal adsorption has been studied using two different techniques: either by atomic absorption spectrophotometric measurements on the metal solution after flocculation or by potentiometric measurements with amalgam electrodes in order to follow the entire complex formation.

Materials and Methods

Four different adsorbing agents were studied with respect to their metal complexing capacity.

1. *Zoogloea ramigera* 115 (ATCC 25935). The organism was maintained on Trypticase Soy Agar (BBL 11043). The medium for suspended growth of the organism had the following composition (g/l): glucose, 25; K_2HPO_4 , 2; KH_2PO_4 , 1; NH_4Cl , 1; $MgSO_4 \cdot 7H_2O$, 0.2; yeast extract (Difco), 0.01. Glucose and $MgSO_4 \cdot 7H_2O$ were autoclaved separately.

The organism was cultivated in a 3-liter fermentor at 26°C. Agitation was 800 rpm and aeration was maintained at 0.5 VVM. Maximum biomass concentration (17.5 g/l) was obtained after 120 hr of culture. The biomass was harvested and stored in a deep-freeze until needed for metal adsorption experiments.

2. *Pseudomonas denitrificans* (ATCC 13876). The organism was cultivated, harvested, and washed according to Nilsson et al.¹³. The wet biomass obtained was used for metal uptake experiments.

3. Carboxymethylcellulose, CMC. Three different modifications of CMC were used for metal uptake experiments, two purchased from Hercules

(Wilmington, Delaware, USA) designed 7MF and 4M6F and one from BDH Chemicals, England. The CMC's used had the following degrees of substitution: 7MF 0.65-0.90, 4M6F 0.38-0.55 and the CMC obtained from BDH 0.7 - 0.8. The polysaccharides were separately dissolved in distilled water under vigorous stirring to give concentrations of 15 g/l. The viscous solutions were stored overnight in the refrigerator before the ability to adsorb metal ions was tested.

4. Polyacrylic acid. The polyacrylic acid prepolymer was Fluka medium viscous (M_w $5 \cdot 10^5$ - $1 \cdot 10^6$). Solutions (0.1 and 0.5% by weight) were prepared in the following way. The prepolymer was dissolved in sodium hydroxide solution and transferred to a cation exchange resin (Dowex 50 WX8) saturated with hydrogen ions. The eluate was collected in a volumetric flask and distilled water was added.

Metal Adsorption

The adsorption of metal ions by the agents was studied by means of different techniques¹¹.

I. Adsorption of copper(II) ions by *Z. ramigera*, *P. denitrificans* and CMC was studied with the following technique:

A metal salt solution was prepared from $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. The metal solution was transferred to a reactor which was equipped with a glass electrode connected to a pH-meter, and a titrator in order to allow control and adjustment of pH. The bacterial cells or the CMC was added to the metal solution. The final volume of the metal solution and the adsorbing agent was 200 ml. All experiments were performed at room temperature. The pH in the mixture was adjusted to 5.5 with 0.05 M sodium borate. The mixture was equilibrated for 15 min with continuous stirring. The exposure was terminated by centrifugation at $11,400 \times g$ for 10 min, which thoroughly separated the bacterial biomass or the CMC from the metal solution. The amount of metal adsorbed by the actual agent was determined by measuring the residual metal concentration in the supernatant. The metal analyses were performed by using an atomic absorption spectrometer (Varian AA 5).

II. The adsorption of copper(II) ions and cadmium ions by polyacrylic acid and by bacterial polysaccharide produced by *Z. ramigera* was studied as a function of pH using a potentiometric technique^{11,14}. A solution containing copper(II) ions (or cadmium ions) and polymer was titrated with a sodium hydroxide solution. pH was measured with a glass electrode (Radiometer GK2402c) and the free metal ion concentration by means of an amalgam electrode. The measurements were performed both in 0.10 M NaClO₄ and without ionic medium.

Results

The measured metal uptake capacity of *Z. ramigera*, *P. denitrificans*, polyacrylic acid and the three different types of CMC are presented in Table 1. When exposed to the copper solution, the three types of CMC behaved differently. Two of the CMC's tested formed large spherical beads (3-8 mm) when mixed with the copper solution. The third CMC, marked 4M6F, precipitated in a manner similar to *Z. ramigera*. The bacterial suspension containing *P. denitrificans* showed no signs of floc formation. By using polyacrylic acid, flocs were obtained although they were smaller than those formed with *Z. ramigera*. The copper adsorption by biomass produced by *Z. ramigera* is significantly higher than the adsorption by the other agents tested.

The uptake of copper and cadmium by polyacrylic acid as a function of pH was calculated from the potentiometric measurements. The results are represented in Figs. 1 and 2. Within the experimental accuracy, the measurements without ionic medium and with 0.10 M NaClO₄ gave coinciding results. A comparison between polyacrylic acid and *Z. ramigera* with respect to adsorption of copper and cadmium shows an important difference. At pH = 5.5, *Z. ramigera* adsorbs substantial amounts of copper but practically no cadmium¹¹. From Fig.1 and 2, it is obvious that polyacrylic acid adsorbs both copper and cadmium at pH = 5.5 although the amount of copper is comparatively higher.

Discussion

Metal removal experiments with the bacterial species used show that *Z. ramigera* adsorbs metal ions much more efficiently than *P. denitrificans*.

The genus *Pseudomonas* has been reported to accumulate substantial amounts of uranium. According to Strandberg et al., *P. aeruginosa* accumulates uranium intracellularly in a rapid process insensitive to environmental conditions². Friedman et al. claim that the ability to accumulate metals is not attributed to the genus *Zoogloea* in general but rather to certain species within the genus *Zoogloea*¹⁵.

Our results show in any case that the species *P. denitrificans* is not very efficient in accumulating copper. Another drawback exhibited by *P. denitrificans* is that flocs are not formed when it is exposed to a metal solution. Separation of biomass from a treated metal solution is greatly facilitated if flocs are formed. Thus, a microorganism suitable for metal uptake should not only exhibit a high metal uptake capacity, but also should be able to form large aggregates upon exposure to metal ions.

From our experiments, it is further evident that the metal accumulation capacity of *Z. ramigera* is superior to that of carboxymethylcellulose. However, CMC is a rather effective agent for metal adsorption. Like *Z. ramigera*, CMC also forms large flocculent aggregates upon exposure to metals, thereby facilitating separation of bound metal from the solution. We find no correlation between degree of substitution of cellulose (and thus the number of binding sites available) and the amount of copper adsorbed to the gum. The considerable difference between copper and cadmium adsorption exhibited by *Z. ramigera* is not shown by polyacrylic acid. Thus, for separation of these two metals, *Z. ramigera* is far more suitable than polyacrylic acid.

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Adsorbing agent		Adsorbed copper
	Conc. (g dwt/l)	(mg Cu/g ads. agent)
CMC (Hercules 7 MF)	0.75	69
CMC (Hercules 4M6F)	0.75	88
CMC (BDH)	0.75	80
<i>P. denitrificans</i>	6.25	11
<i>Z. ramigera</i>	0.83	270

Table 1. Comparison of copper uptake by biomass from *Zoogloea ramigera* with other adsorbing agents. The copper concentration was 0.74 - 0.54 g/l; equilibrium pH was 5.5 and the contact period was 15 min.

Adsorbing agent	Conc. of biomass (g dwt/l)	Copper adsorbed		Cadmium adsorbed	
		(%)	mg Cu/g biomass	(%)	(mg Cd/g biomass)
<i>Z. ramigera</i>	0.50	56	132	1	4
Polyacrylic Acid	0.80	76.5	113	54	148

Table 2. Comparison of copper and cadmium uptake by *Z. ramigera* and polyacrylic acid. The data were obtained from potentiometric measurements. The copper concentration was 1.87 mM; the cadmium concentration was 2.28 mM and pH = 5.5.

Legends to figures

Figure 1. Bound copper ($(C_{Cu} - [Cu^{2+}])/C_{Cu}$) to polyacrylic acid as a function of pH.

$C_{Cu} = 2.5 - 2.0 \text{ mM}$

□ polyacrylic acid concentration 1.05 - 0.75 mg/l

○ polyacrylic acid concentration 0.21 - 0.18 mg/l

☆ no polyacrylic acid added

Figure 2. Bound cadmium ($(C_{Cd} - [Cd^{2+}])/C_{Cd}$) to polyacrylic acid as a function of pH.

$C_{Cd} = 2.5 - 2.0 \text{ mM}$

□ polyacrylic acid concentration 1.05 - 0.75 mg/l

○ polyacrylic acid concentration 0.21 - 0.18 mg/l

☆ no polyacrylic acid added

FIG. 1

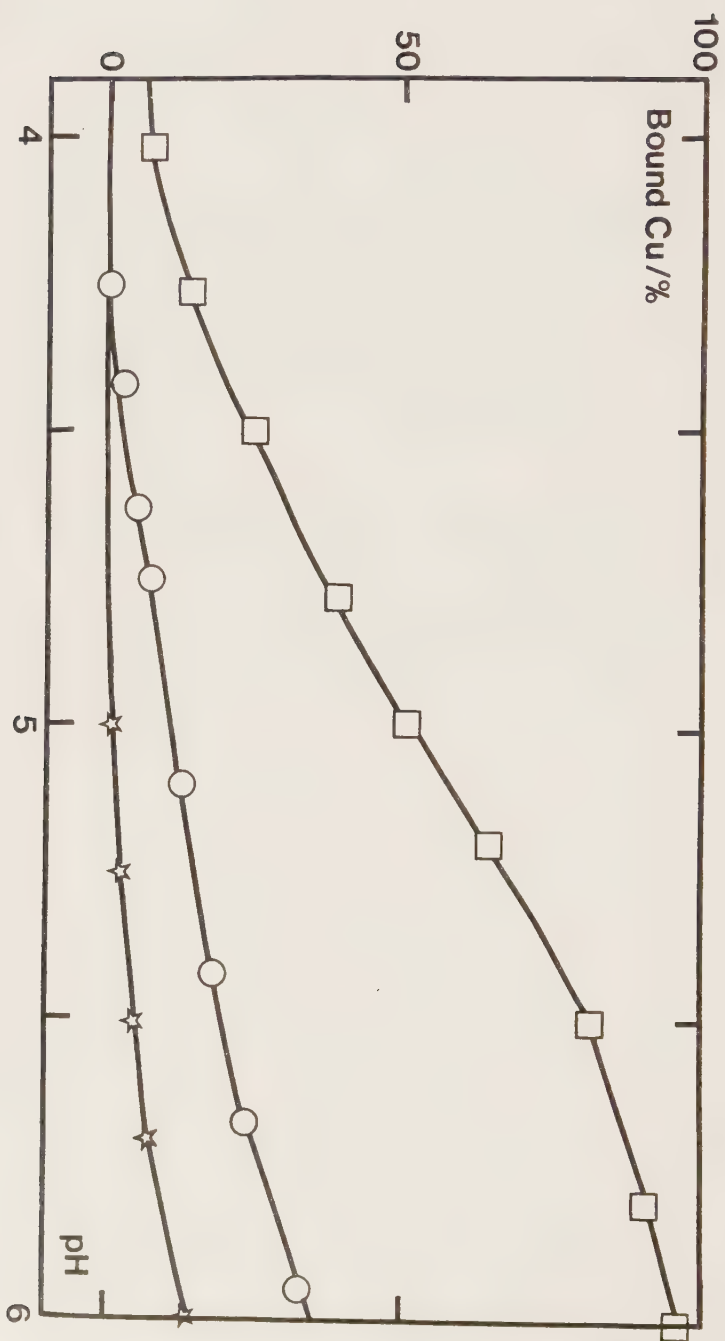
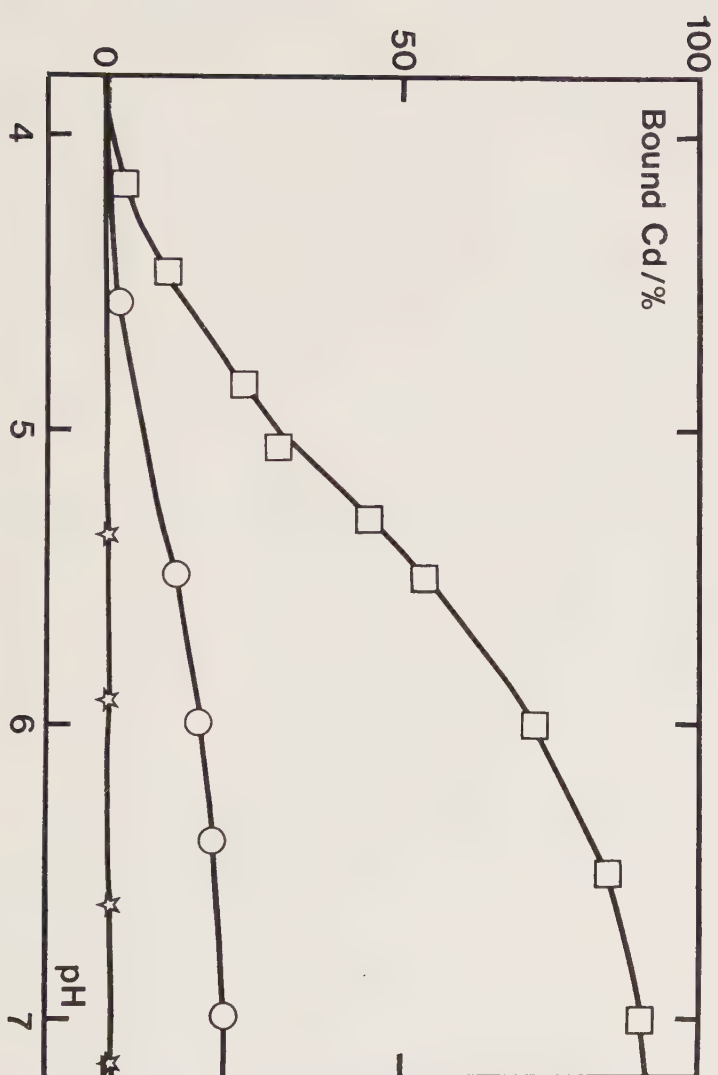


FIG. 2



Development of a continuous process for metal accumulation by *Zoogloea ramigera*.

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DEVELOPMENT OF A CONTINUOUS PROCESS FOR METAL ACCUMULATION BY *ZOOGLAEA RAMIGERA*

ABSTRACT

Biomass, mainly consisting of an acidic polysaccharide produced by *Zoogloea ramigera*, has been used as an adsorbing agent in a continuous process for the recovery of metal. The adsorption of copper has been studied with respect to retention time, biomass concentration and reaction pH, in order to determine the optimal conditions for copper recovery. The results indicate that the uptake of copper is rapid and efficient. About 0.17 g Cu is adsorbed per g biomass within 10 min. At high biomass concentrations, the total amount of copper removed from solution is high, but the specific amount of copper adsorbed to biomass is low. The biomass exhibits a higher adsorptive uptake at low concentrations.

INTRODUCTION

Microbially assisted removal and accumulation of metals from solutions have, during the last decade, shown results which have stimulated continued and expanding research within this area.

Novel processes for the accumulation of metal ions from dilute solutions have been proposed. Bacteria, yeast and algae have been successfully used as adsorbing agents for heavy metals. Interaction between metals and microbial cells can occur through the adsorption of metals to cell surfaces, through metabolically assisted accumulation within the cell or as metallic complexes with extracellular microbial metabolites.¹ For instance, bacterial extracellular polysaccharides in activated sludge have an extensive complexing capacity for heavy metals.² Physical measurements have shown that acid polysaccharides can function as polyelectrolytes and thus form the basis for metal adsorption.^{3,4} The bacterium *Zoogloea ramigera* produces an extracellular polysaccharide when cultivated in appropriate media.⁵ The polysaccharide is composed of galactose, glucose and pyruvate.⁶ The biomass produced can act as an efficient adsorbing agent for heavy metal ions.^{7,8}

We have previously described a process which worked on batchwise flocculation of metal solutions by adding microbial biomass. The biomass, once used, can be regenerated, which permits recycling.⁷

In this paper, we report on a continuous process for metal uptake. The backbone of the process, the metal uptake phase, has been separately developed to permit operation under continuous conditions. The influence on retention time, biomass concentration and pH on metal accumulation capacity have been studied.

MATERIALS AND METHODS

Organism and culture

Zoogloea ramigera strain 115 (ATCC 25935) was used throughout this study. Media and culturing conditions were as described previously (Norberg, Enfors 1982). The biomass was produced in batch cultivations, harvested

and stored in a deep freeze until needed. The dry weight of the biomass was 22 g/l. Due to its pseudoplastic flow behaviour, it was easily pumped.

Metal uptake apparatus

The experimental set-up is shown in Fig. 1. The reactor was equipped with a baffle in order to achieve turbulent flow. A pH-electrode connected to a pH-meter was used to record the acidity of the solution. A titrator joined to the pH-meter was used to adjust pH to preset values. A conductivity electrode connected to a peristaltic pump was used to keep the liquid volume of the reactor at a constant level of 200 ml.

Mixing was achieved with the use of a magnetic stirrer. The stirring rate was 700 rpm.

The metal solution was stored in a 10-liter glass vessel. A peristaltic pump regulated the flow rate of metal solution to the reactor.

Biomass for metal adsorption was supplied from a storage vessel (500 ml). A continuously operating peristaltic pump transferred biomass from the storage vessel to the reactor.

The pumps regulating the inflow of biomass and metal solution to the reactor were calibrated in order to permit control of the actual flow rate. Tygon plastic tubing (Norton, Akron, Ohio, USA) was used in order to minimize flow variations due to material exhaustion (inconsistency).

Copper was supplied as $\text{CuCl}_2 \times 2\text{H}_2\text{O}$. All experiments were performed at room temperature.

Effect of biomass concentration

The biomass concentration in the reactor was varied from 0.44 to 3.08 g/l (dry wt.) at a constant retention time of 8 min. The used metal solution contained 0.60 g Cu^{2+} /l and the pH was adjusted to 5.50 by adding 0.05 M Na-borate.

The sum of the inflows to the reactor was kept constant at 25 ml/min. When the flow rate of biomass was 0.5 ml/min., corresponding to a dry weight of 0.44 g/l in the reactor, the metal flow was adjusted to 24.5 ml/min. After 15 min., a sample (15 ml) was withdrawn from the reactor. The sample was centrifuged (11,400 x g, 10 min.) and the resulting supernatant was analyzed with respect to remaining soluble copper by using an atomic absorption spectrometer. The flow of biomass to the reactor was then increased to 1.0 ml/min and the flow of metal solution was decreased to 24 ml/min.

The system was allowed to equilibrate for 15 min. Thereafter, another sample was withdrawn and treated as described above. The biomass flow was increased in increments of 0.5 ml/min up to 3.5 ml/min, and the flow of metal solution was adjusted simultaneously in order to maintain a total flow of 25 ml/min. A sample for metal analysis was taken after each adjustment of biomass flow. Two separate experiments were performed. The data presented is the mean value of these two runs.

Effect of pH on continuous uptake of copper

A copper solution (0.30 g/l) was pumped through the reactor at a flow rate of 19 ml/min. Simultaneously, a biomass flow of 1 ml/min. was maintained through the system. The total flow of 20 ml/min. gave a retention time of 10 min. at a biomass concentration of 1.1 g/l (dry wt.) in the reactor. These conditions were kept constant throughout the study.

The experiment was initiated by starting the flow of biomass and copper solutions. The system was allowed to equilibrate, which resulted in a pH-value around 4.75. Addition of NaOH (0.5 M) raised pH to a preset value of 4.80. After 15 min., a sample was withdrawn from the reactor. The same procedure was repeated with successive increases of pH in increments of 0.3 pH-units. After each adjustment, the system was allowed to equilibrate for 15 min. after which a sample was taken for metal analysis. The final sample was withdrawn at a pH value of 7.5. Metal adsorptions at pH-values below 4.75 were studied by titrating to pre-determined pH levels with HCl (0.5 M). The pH-values studied with respect to metal uptake were 4.45, 4.15, 3.85, 3.55 and 3.25. Samples were withdrawn at each level and treated according to the conditions described above.

Influence of retention time

A copper solution (0.58 g/l) was mixed with biomass (22 g/l) in the metal uptake reactor. Both ingredients were constantly pumped through the reactor. The following retention times were studied: 4, 5, 8, 10 and 20 min. This was performed by adjusting the total flow rate to 50, 40, 25, 20 and 10 ml/min. The flow rate of biomass was 5% of the total inflow rate which assured a constant biomass concentration of 1.1 g/l (dry wt.) in the reactor throughout the experiment. After each flow adjustment, 15 min. were allowed to elapse before a sample was withdrawn from the reactor. The sample was centrifuged and analyzed with respect to copper content as described above.

RESULTS

The complete process set up for the continuous extraction of copper from aqueous solutions by adsorption to microbial biomass is shown in Fig. 1. The results show that the biomass concentration strongly affected the amount of copper removed from an aqueous solution (Fig. 2). However, the amount of copper adsorbed per g of biomass decreased when the biomass concentration in the reactor increased (Fig. 2).

At higher biomass concentrations, the aggregates formed during the flocculation tended to agglomerate. These large aggregates (0.5 - 1.5 cm) were not continuously removed from the reactor, as expected, but rather tended to accumulate in the reactor due to their larger size. This behaviour disturbed the equilibrium within the reactor volume and could explain the irregularities in the metal adsorption data obtained at biomass concentrations above 1.5 g/l (dry wt.). A possible solution to the agglomeration problem could be the adjustment of stirring rate in proportion to biomass concentration. High shear rate facilitates the break up of large aggregates into smaller particles.

Removal of copper from an aqueous solution was more efficient with increasing pH (Fig. 3). However, at higher pH-values, only part of the copper removed is due to adsorption to bacterial biomass since the formation of copper hydroxide is initiated above pH 5.0. The precipitate formed seems to be trapped within the polymer matrix.

The effect of retention time on the adsorption of copper to biomass was marginal (Table 1). The results indicate that the rate of metal uptake is rapid and thus suitable for process application. However, using centrifugation to separate the aggregated biomass from the metal solution automatically prolongs the time of exposure.

DISCUSSION

Microbial removal of heavy metals from aqueous process effluents, based on continuous modes of operation, has been used for different purposes. Continuous heavy metal ion removal from a bacterial leaching solution using a mixed culture of sulfate-reducing bacteria exhibited promising results.⁹

A continuously working process for the simultaneous removal of nitrate and heavy metals by using immobilized *Pseudomonas fluorescens* as an adsorbing agent for different cations, has also shown fruitful results.¹⁰

In our previous reports on metal accumulation by *Z. ramigera*, all results were based on batchwise operations.^{7,11} If the copper uptake capacity of biomass in batch and continuous operating systems is compared, it is clearly demonstrated that the batch system is superior. The amount of copper adsorbed during continuous extraction was approximately half the amount adsorbed during batchwise operation. The reason for this difference in metal uptake capacity, can be found in the way the biomass is introduced into the systems. During batch operation, the biomass is added to the metal solution and freely allowed to dissolve or form a suspension in water. The biomass strongly attracts the water molecules. If the concentration of polysaccharide is sufficient, it forms a network throughout the solution. The metal cations present in the system may diffuse freely into this network and labile interactions between cations and charged sites on the polysaccharide can occur. When the pH is increased, stronger interactions between ions and biomass occur until finally the polymer flocculates or precipitates. At the beginning of precipitation, polysaccharide molecules tend to react more with other polysaccharides and cations than with water. Complexing of oppositely charged ions continues until a point is reached at which the polymer is

precipitated. The precipitates (or flocs) formed are cohesive and stringy.

Batchwise exposure of heavy metals to microbial biomass is based on other fundamentals than a system for continuous retrieval. In the continuous system described here, biomass is supplied through a port at the bottom of the reactor. The forces evolved through agitation pull the biomass out into the reactor and flocculation is completed within seconds. During this time, the polymer is not allowed to form a network throughout the solution and thus facilitate an even distribution of cations within the polymer matrix before flocculation is initiated. In this case, it seems possible to assume that interaction between cations and biomass (including adsorption of cations to both cell surfaces and biopolymer) takes place only at the outermost region of the biomass particles. Due to the dehydration of the surface layers of the biomass during the floc formation, cation penetration through these layers may be strongly reduced or totally blocked. The inner part of the biomass particles will thus not be available for metal adsorption. This dissimilarity between the systems could be the reason for the great difference in metal uptake capacity.

In the experiment concerning the effect of pH on uptake of copper by biomass, no reliable estimation of soluble copper concentration could be performed at pH values below 4.8 with the measuring method used in this study. However, we have earlier shown by using a potentiometric method, that copper is not adsorbed to the polymer at pH-values below 4.5.⁷

A conclusion to be drawn from this study is that a continuous process can be used as a tool in order to find the optimum conditions for metal accumulation. If, for example, five different metal ions in mine drainage water are of economical interest (and assuming that all can be concentrated by biomass produced by *Z. ramigera*), each metal can be tested in the system described above. Vital parameters regarding metal adsorption can rapidly and conveniently be screened and the optimum conditions for each metal can thus be determined. This information will show whether or not selective removal is possible. However, for application purposes, a process based on batchwise operation is, to date, more favourable than a continuous process.

Acknowledgements

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Table 1. Effect of retention time on adsorption of copper to biomass.

Retention time (min.)	Copper conc. in solution (g/l)	Copper in biomass (g/g dry wt.)
4	0.37	0.19
5	0.39	0.17
8	0.41	0.15
10	0.41	0.15
20	0.40	0.16

Legends to figures

Fig. 1 Experimental set-up for the continuous extraction of heavy metals from aqueous solution.

Fig. 2 Effect of the biomass concentration on the uptake of copper in a continuous system.

Fig. 3 Effect of pH on the uptake of copper.

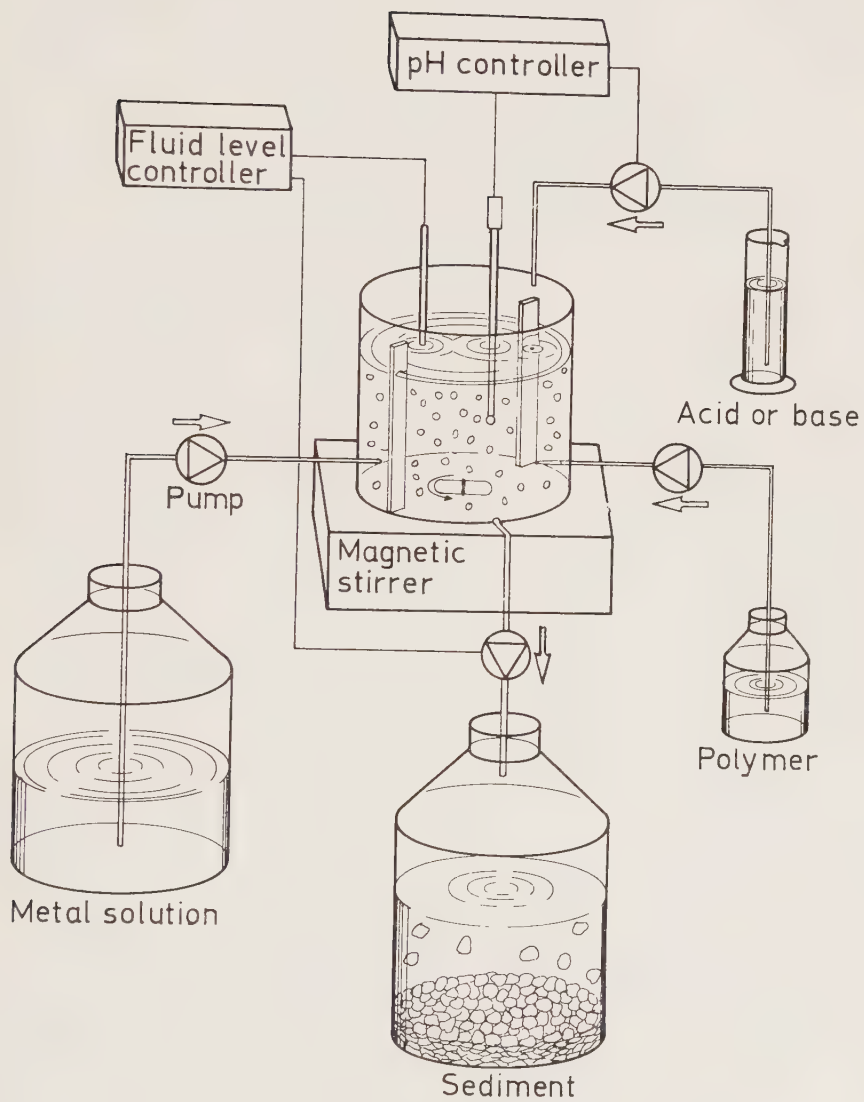


Fig.1

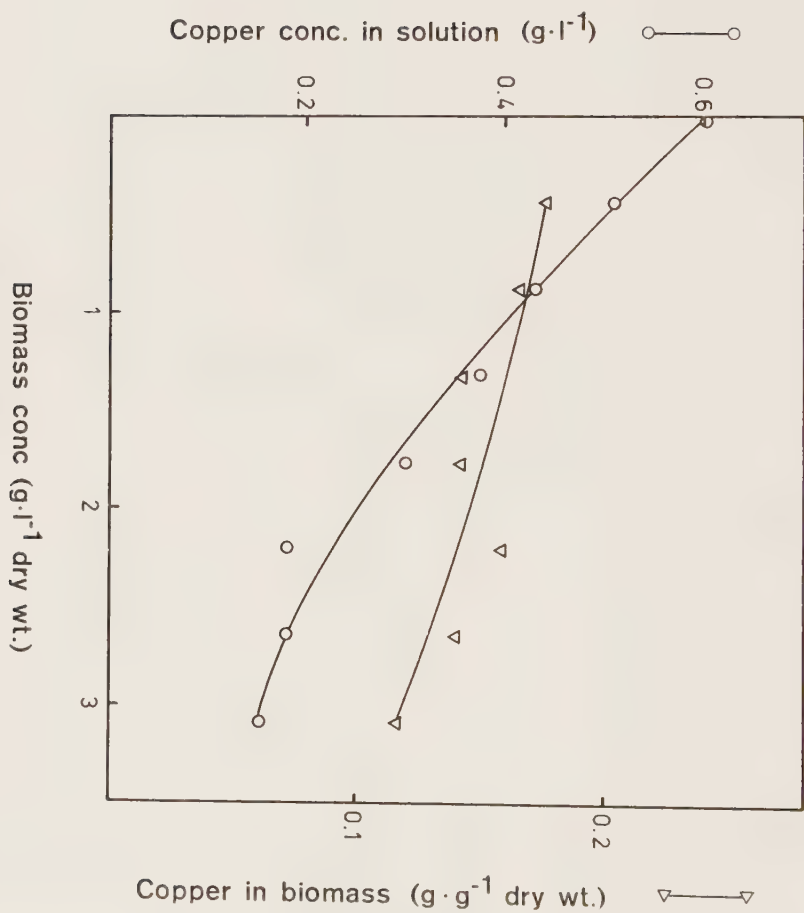


Fig. 2

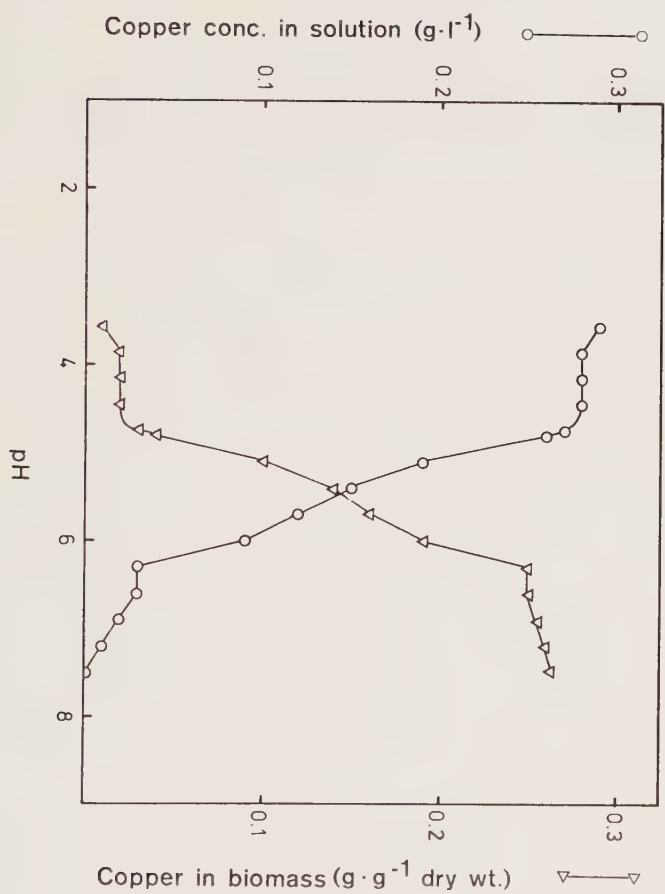


Fig. 3

Tillverkning och användning av biopolymerer.

Som en konsekvens av industriell belastning, handelsgödsel och sur urlakning har koncentrationerna av lösta toxiska metaller ökat avsevärt under de senaste årtiondena, vilket medfört svåra miljöproblem. Det finns ett behov av nya metoder för att lösa dessa problem.

5 Föreliggande uppfinning gäller ett nytt sätt att ta hand om lösta metaller genom användning av nya typer av polysackarider, framställda genom en ny metod. Samtidigt har förfaranden utvecklats för användning av de nya polysackariderna på flera andra områden.

10 Genom vår uppfinning har en ny metod att framställa polysackarider utvecklats som ger överlägsna egenskaper vid användning för bindning av metaller. Metoden är dessutom ekonomiskt fördelaktig.

15 De polysackarider som framställts enligt vår metod är anmärkningsvärt resistenta mot nedbrytning. Dessa polysackarider och bakteriella cellväggskomponenter har en hög täthet av anjoniska laddningar och interagerar kraftfullt med katjoniska metalljoner och kan härigenom ackumulera stora kvantiteter av bunden metall.

20 Vid framställningen av polysackariderna har vi använt bakterien *Zooglera ramigera*, som visat sig vara en mycket god producent av en extracellulär heteropolysackarid, vars egenskaper gör att den med fördel kan användas för adsorption av tungmetalljoner.

25 *Zooglera ramigera* syntetiserar polymeren i en process, som delvis är knuten till tillväxten. Under odlingens initiala del är polymeren bunden vid cellväggen, varvid viskositeten hos odlingsvätskan ökar snabbt. Ultraljudsbehandling av biomassan från odlingens tidigare skede leder också till att kapseln lösgörs.

Bakteriens tillväxtfas avslutas inom 20 h men glukoskonsumtionen fortsätter att ligga högt långt därefter. Under de första 70 h odlingstimmarna är polysackariden fortfarande bunden vid cellväggen

i form av en kapsel men efter ca 90 h förekommer polysackariden också i odlingsmediet, vilket avläses genom ökning av viskositeten. De slutliga värdena på inre friktion ligger på 800-1000 cP. Efter att viskositeten nått sitt maximum sker ingen nedgång, vilket indikerar att ingen nedbrytning av polysackariden sker.

Kol-kväve-kvoten är viktig för att begränsa mängden bildad cellmassa och för att styra större delen av tillsatt socker till polysackarid. Substratets C/N-kvot bör ligga mellan 30-40. Den initiala koncentrationen av N bör ligga kring 0,25 g/l.

10 Inpumpningen av kolkälla under polymerproduktionssteget bör regleras så att en jämn nivå glukos finns tillgänglig för cellerna under hela odlingsperioden. Den tillförda glukosen bör utnyttjas till fullo. Inpumpningshastigheten regleras i omvänt förhållande till partialtrycket av CO_2 i utgående luft.

15 Upparbetning av polymeren sker genom att tillsätta två- eller trevärda katjoner, så att polymeren flockas ut. Polymeren separeras sedan från odlingsvätskan genom centrifugering.

Polymeren kan därefter ytterligare upprenas på traditionellt sätt genom att använda alkohol t ex etanol.

20 Genom vår metod bibehålles hög viskositet under hela produktionsfasen genom att kapslarna hålles intakta. Odlingsförhållandena är skonsamma. Odlingsmediets sammansättning är viktig.

Metoden kommer att närmare beskrivas genom nedanstående exempel.

Exempel 1. Batchförsök utan feeding.

Fastställande av optimal C/N-kvot på medium för produktion av extracellulär polysackarid med *Z. ramigera* stam 115 har skett i separata batchodlingar. Vid försöken har en glukoskoncentration kring 25 g/l använts, genom att tillsätta varierande mängd kvävekälla ($0,25-4 \text{ g NH}_4\text{Cl/L}$) till mediet vid de olika odlingarna har det visat sig att det optimala utbytet av polysackarid från satsad glukos erhålles vid $0,5-2 \text{ g NH}_4\text{Cl/L}$. Den totala odlingstiden är 36 till 60 timmar.

Exempel 2. Batchförsök med feeding.

Optimal C/N-kvot på medium när polysackarid produceras i ett batchsystem som tillföres ytterligare kolkälla genom inpumpning av glukos har fastställts till mellan 10 och 25. Genom den högre kvävehalten under den initiala batchprocessen kan en högre cellmassa byggas upp. När den ursprungliga glukosen förbrukats startar inpumpning av glukos. Den befintliga cellmassan omvandlar effektivt den tillförda glukosen till polymer. Dosering av glukos regleras automatiskt med hjälp av signalvärde från CO_2 -analysator som mäter halt CO_2 i utgående gas från fermentor. Inpumpning av glukos startar när signalen sjunker under förinställt börvärde. Behövlig odlings-tid är 36-54 timmar.

Exempel 3.

Optimal halt löst O_2 i medium har fastställts i batchodlingar. Luftningen har varierats i separata försök, omrörningen som också påverkar halten löst O_2 har hållits konstant vid 800 rpm. Om luftflödet genom fermentorn hålles vid $0,25-1,5 \text{ VVM}$ (volym luft per volym medium och minut) erhålles de bästa betingelserna för konvertering av glukos till polymer. Halten löst O_2 underskred aldrig 20 % av mättnadsvärdet under de försök som gav god omvandling av glukos till polymer.

Exempel 4.

Ett viktigt spårämne vid polymerproduktion med *Z. ramigera* har visat sig vara järn. Tillsats av järn skall ske under bakteriens tillväxtfas. Detta utföres så att en järnlösning (ca 0,01 M) doseras in i fermentormediet samtidigt som fermentormediets surhetsgrad regleras. Inpumpning av järn sker således automatiskt och är proportionell till bakteriens tillväxthastighet.

Alla försök beskrivna här är utförda med järntillsats till mediet, vilket delvis har inneburit att den totala processtiden har kunnat förkortas från 100 till 36-60 timmar.

Exempel 5.

Upparbetning av den viskösa produkten kan ske genom att trevärd aluminium tillsätts till den bildade bakterieprodukten. Genom reglering av Al^{3+} -koncentrationen och lösningens surhetsgrad till bestämda värden erhålles en flockad produkt. Flockarna kan separeras från den omgivande vattenfasen genom centrifugering. Den erhållna polymeren håller en lägre vattenhalt (5-50 viktprocent) än den ursprungliga.

Exempel 6.

Beskrivning av odling med feeding av glukos där optimal C/N-kvot fastställdes.

Försök 1: Initial glukosconc 25 g/l, kväveconc var 0,26 g/l. Mediet innehöll förutom dessa komponenter (g/l); K_2HPO_4 , 2; KH_2PO_4 , 1; $MgSO_4 \times 7H_2O$, 0,2; jästextrakt (Difco), 0,01. Dosering av $FeCl_3 \times 6H_2O$. Den ursprungligt tillsatta glukosen förbrukades inom 50 h, tillsats av ytterligare glukos (5 g/l) till fermentormediet gav som resultat en ökad polymerconc, men polymersyntesen var långsam.

Slutlig polymerconc; 18 g/l
 Slutlig cellmassa; 2,3 g/l

Försök 2: Kvävekonc i medium 0,52 g/l i övrigt som i försök 1.

Cellmasseproduktion avstannar efter 14 timmar, ursprunglig glykos slut efter 22 timmar. Inpumpning av ny glukos gav en snabb omvandling av glukos till polymer.

Vid avslutad odling (48 timmar) var polymerkonc 32 g/l, cellmassekonc var 4,3 g/l.

Försök 3: Kvävekonc i medium 1,04 g/l i övrigt som i försök 1.

Produktion av cellmassa avstannar efter 18 timmar, initialt tillsatt glukos slut efter 20 timmar. Glukosin-pumpning gav en mycket snabb omvandling av glukos till polymer, men stora mängder glukos gick här förlorad som

CO₂. Vid odlingens slut (45 timmar) var polymerkonc 25 g/l, cellmassan uppgick till 9,8 g/l.

Den heteropolysackarid som bakterien Zooglerea ramigera syntetiserar är sammansatt av glukos, galaktos och pyruvat och skiljer sig från tidigare kända polysackarider. Polymeren är grenad och dess molvikt varierar men torde ligga vid ca $10^5 - 10^6$ dalton. Mängden pyruvat i polymeren är beroende av odlingsbetingelserna, 5% uppnås lätt.

Följande applikationer på biopolymeren har undersökts:

1. Utvinning av värdefulla metaller ur utspädda lösningar. Mest intressant framstår anrikning av koppar, zink och vanadin ev molybden ur gruvvatten. Stora kvantiteter vatten med höga halter av framför allt koppar och zink renas idag partiellt utan att metallerna tillvaratas. I dagens läge finns ingen effektiv teknik för att tillvarata denna resurs. Vår basprocess för metallackumulering kan här användas.
2. Avlägsnande av toxiska metaller från avloppsvatten. Här avses skräddarsydda processpaket för industrier med tungmetallproblem. Galvaniseringsindustrier, ytbehandlare, kretskortstillverkare, ackumulatortillverkare eller företag inom destruktionsbranschen är lämpliga målgrupper. Eftersom ingen

specificitet krävs bör vår process kunna integreras tämligen problemfritt.

3. Anrikning av specifik metall ur vattenlösning innehållande ett flertal metaller. Vissa vatten kan innehålla en värdefull metall (uran, silver, vanadin eller kvicksilver) och många andra metaller som i sammanhanget anses ointressanta ur koncentreringsyfte. Genom vidareutveckling av redan funnen teknik för specifik anrikning kan fler metaller anrikas separat med tolerabel störning från ej intressanta metaller.
4. Hindra vattenavdunstning från växtfröer under gröningsprocessen - pelletering. Belägga fröer med ett skikt polymer så att fröerna får uniform storlek, vilket underlättar såningsprocessen. Polymeren kan också verka som ett skydd mot insektsangrepp, svampinfektion och frostsador så länge fröet inte börjat gro.
5. Koncentrering av toxiska organiska ämnen. Vissa organiska ämnen kan koncentreras med polymeren. Vid destruktion (förbränning) av vattenlösliga organiska ämnen önskas en så hög koncentration av kemikalien som möjligt. Detta kan åstadkommas med vår polymer, vilket då avsevärt förbilligar förbränningsprocessen.
6. Avsaltning av havsvatten - delvis eller tillräckligt för att vattnet skall kunna användas för bevattningsändamål. De vanligast förekommande jonerna i havsvattnet är Na^+ , Mg^{2+} , K^+ , Ca^{2+} , Cl^- och SO_4^{2-} . Några eller någon av dessa joner kan reduceras.
7. Deposition av spårelement i konstgödsel eventuellt i rotklumpar för småplantor. Spårämnen bundna till vår polymer frigörs långsamt i takt med att polymeren naturligt brytes ned i jorden. Spårämnena blir då tillgängliga för växten i lagom koncentration och vid den tid i tillväxten då de som bäst behövs. Selen t ex verkar intressant här.

8. Minska metallhalten i dricksvatten. Främst gäller detta bly, koppar och kadmium. Ett vattenfilter med en fast polymermatrix över vilken vattnet strömmade och samtidigt befriades från tungmetaller. Ny teknik krävs, stor selektivitet efterfordras med avseende på inbindning av metall till polymer.
9. Läkemedelsinkapsling med polymer. Vissa läkemedel tål ej magsafter och förstörs därför när de passerar magsäcken. Upptagning av läkemedlet sker däremot alltid i tarmkanalen. Om läkemedlet inkapslades i polymer så att det ej exponerades för den sura miljön i magen, men löstes upp enzymatiskt i tarmen är detta av stort värde.
10. Flockningskemikalie i reningsverk. För flockning av aerobt renat vatten användes olika polymera material. Flera av dessa polymerer är dyra och en del hälsovådliga. Stora mängder flockningskemikalier användes idag vid landets reningsverk.

PATENTKRAV.

1. Mikrobiellt framställda polysackarider, k ä n n e t e c k n a d e av att de är sammansatta av glukos, galaktos och högre halt av pyruvat.
- 5 2. Förfarande för att framställa polysackarider enligt patentkrav 1, k ä n n e t e c k n a t av, att bakterien Zooglerea ramigera användes vid framställningen.
3. Förfarande enligt patentkrav 2, k ä n n e t e c k n a t av att glykos, kvävekälla och mineralämnen tillföres på ett i förväg programmerat sätt i proportion till produktbildnings-
10 hastigheten.
4. Förfarande enligt patentkraven 2-3, k ä n n e t e c k n a t av, att järn i form av järnlösning tillföres under bakteriens tillväxtfas.
5. Förfarande enligt patentkraven 2-4, k ä n n e t e c k n a t av, att produkten upparbetas genom att trevärt aluminium tillsättes till den bildade bakterieprodukten.
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6. Användning av polysackarid^{med cellmassa}er enligt patentkrav 1, k ä n n e t e c k n a d av att metaller ur utspädda lösningar anrikas med hjälp av polysackariden.
- 20 7. Användning av polysackarid^{med cellmassa}er enligt patentkrav 1, k ä n n e t e c k n a d av, att växtfröer beläggas med ett skikt av polysackarid för att hindra vattenavdunstning under gröningsprocessen, underlätta såningsprocessen och utgöra skydd mot insektsangrepp, svampinfektioner och frostskador.
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8. Användning av polysackarider^{med cellmassa}venligt patentkrav 1, k ä n-
n e t e c k n a d av, att läkemedel inkapslas med poly-
sackariden och skyddas mot den sura miljön i magen.
- 5 9. Användning av polysackarider^{med cellmassa}venligt patentkrav 1, k ä n-
n e t e c k n a d av, att spårämnen bindas till poly-
sackariden så att spårämnena långsamt frigöras och blir
tillgängliga för växterna i takt med att polysackariden
bryts ner i jorden.
- 10 10. Användning av polysackarider^{med cellmassa}venligt patentkrav 1, k ä n-
n e t e c k n a d av, att polysackariden tillsättes
vatten som flockningsmedel.

Sammandrag.

Uppfinningen gäller nya polysackarider uppbyggda av glukos, galaktos och pyruvat. Framställningen sker med hjälp av bakterien Zooglerea ramigera under noggrant reglerade betingelser med tillsats av järn och med användning av trevärt aluminium vid utvinningen. De nya polysackariderna har hög täthet av anjoniska laddningar och interagerar kraftfullt med metalljoner.

Polysackariderna kan bl.a. användas för anrikning av metaller ur vattenlösningar, för pellettering av fröer, för deposition av spår-element, för inkapsling av läkemedel och som flockningsmedel vid vattenrening.

